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KINETICS AND SUBSTRATE-BINDING STUDIES  
OF SUCCINYL COENZYME A SYNTHETASE

by



FRANK JAMES MOFFET

A THESIS

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The undersigned certify that they have read, and  
recommend to the Faculty of Graduate Studies and Research,  
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STUDIES OF SUCCINYL COENZYME A SYNTHETASE" submitted by  
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degree of Doctor of Philosophy.



## ABSTRACT

The kinetics of succinyl CoA synthetase can be interpreted in terms of a random sequential ter-ter mechanism. Initial rate kinetic studies from both directions of the reaction indicate that there is a preferred pathway involving initial binding of ATP or ADP to the enzyme followed by random addition of other substrates. Kinetics of ATP  $\longleftrightarrow$  ADP isotope exchange at equilibrium suggest that minor pathways initiated by binding of substrates other than ATP or ADP are also operative. Neither the initial rate kinetic or the isotope exchange results can be reconciled with a significant rate of operation of a ping-pong mechanism despite the known participation of a covalent phospho-enzyme intermediate in the reaction.

Succinyl CoA synthetase is strongly protected from trypsin-catalyzed inactivation by phosphorylation of the active site histidine or by binding of inorganic phosphate. Other substrates have no effect on sensitivity to trypsin. The results can be interpreted in terms of a conformational change on phosphorylation or phosphate binding. It is proposed that this conformational change may result in a negative co-operativity between two active sites in a 140,000 M.W. tetramer.

Studies of the binding of succinate to phosphorylated enzyme in the absence of Mg<sup>++</sup> and ADP to dephosphorylated enzyme in the presence of Mg<sup>++</sup> have been carried out. Only a low stoichiometry of succinate binding is observed under these conditions and the amount of binding is not proportional to specific activity. The binding of ADP is proportional to specific activity and supports the possibility of 2 active sites per tetramer of succinyl CoA synthetase.



Dissociation constants for succinate from the phosphorylated enzyme and  $P_i$  and ADP from the dephosphorylated enzyme have been determined, and are found to be of the same order of magnitude as the  $K_m$  values for these substrates determined from initial rate kinetic data.



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## LIST OF ABBREVIATIONS

ADP	- adenosine 5'-diphosphate
ATP	- adenosine 5'-triphosphate
Aopopcp	- $\beta,\gamma$ -methylene-ATP
CoA	- Coenzyme A
EDTA	- Ethylenediaminetetraacetate
E $\sim$ CoA	- proposed high energy form of SCS containing bound CoA
I	- Intensity
K <sub>d</sub>	- dissociation constant
K <sub>m</sub>	- Michaelis constant
M.W.	- Molecular weight
n	- number of binding sites per enzyme molecule
nm	- nanometers
O.D. <sub>280</sub>	- optical density at 280 nanometers
P <sub>i</sub>	- inorganic phosphate
r	- $\mu$ moles of substrate bound per $\mu$ mole of enzyme
SDS	- sodium dodecyl sulphate
SCS	- succinyl CoA synthetase
succ.	- succinate
succ-CoA	- succinyl Coenzyme A



## LIST OF ABBREVIATIONS (continued)

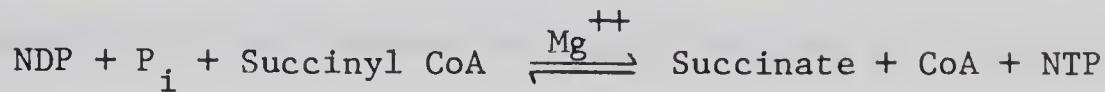
succinyl-P	- succinyl phosphate
TPCK	- L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone
Tris	- tris (hydroxymethyl) aminomethane
$\mu\ell$	- microliter
$V_{max}$	- initial velocity at infinite concentration of substrates
E-P	- phosphorylated SCS



## CHAPTER I

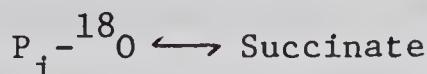
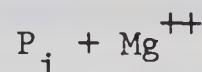
## INTRODUCTION

Succinyl CoA synthetase (also known as succinic thiokinase) catalyzes the reaction:

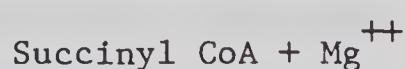


This reaction is the substrate-level phosphorylation step accompanying  $\alpha$ -ketoglutarate oxidation in the TCA cycle and was originally elucidated by Kaufman *et al.* (1) and by Hift *et al.* (2). NTP and NDP represent guanosine or inosine phosphates in the case of mammalian SCS (Succinate: CoA ligase (GDP), E.C. 6.2.1.4) and adenosine phosphates in the case of plant or microbial SCS (Succinate: CoA ligase (ADP), E.C. 6.2.1.5).

Early work with this enzyme was carried out with partially purified preparations from various sources including hog heart and kidney, spinach leaves and Escherichia coli. The enzyme was found to catalyze a number of isotope exchange reactions which are summarized below:

Exchange Reaction:Substrate Requirement:

or





The enzyme was also shown to catalyze a rapid arsenolysis of succinyl CoA requiring  $Mg^{++}$  but independent of nucleotides. This early work, up to about 1962, has been reviewed by Hager (3).

Kaufman, on the basis of the observed ATP  $\longleftrightarrow$  ADP exchange in the absence of other substrates, early proposed an enzyme-phosphate intermediate as part of the succinyl CoA synthetase reaction (4). Such an intermediate is also in harmony with the arsenolysis data mentioned above. The isolation and characterization of this intermediate however awaited the availability of more highly purified enzyme.

Upper (5) in 1964 reported the purification of SCS in high yield from succinate-grown *Escherichia coli* (Crookes strain). He demonstrated the incorporation of radioactivity into the enzyme from ATP- $\gamma$ -<sup>32</sup>P but not from ATP-8-<sup>14</sup>C. Earlier, Boyer and his coworkers had reported the presence of a phosphohistidine-containing component in extracts of mammalian mitochondria (6,7) and subsequently had shown that the phosphoprotein obtained from pig heart mitochondria was closely associated with SCS activity (8). Using Upper's isolation method Kreil and Boyer (9) were able to label highly purified SCS from *E. coli* with <sup>32</sup>P<sub>i</sub> in the presence of succinyl CoA and  $Mg^{++}$ . They then showed that the labelled residue obtained from an alkaline hydrolysate of the protein was phosphohistidine. The recovered residue was subsequently identified as the 3-phosphohistidine isomer (10). In other studies Cha *et al.* (11) demonstrated that partially purified pig heart SCS could incorporate <sup>32</sup>P<sub>i</sub> from either GTP- $\gamma$ -<sup>32</sup>P or from <sup>32</sup>P<sub>i</sub> + succinyl CoA.



The preparative procedure developed by Upper was subsequently modified in P.D. Boyer's laboratory (12). These workers obtained a homogeneous preparation of SCS, with high specific activity, from E. coli, and carried out an investigation of the properties of both the phosphorylated and dephosphorylated forms of the enzyme. A molecular weight of 141,000 was found for the homogeneous preparation by sedimentation equilibrium analysis. The enzyme was found to incorporate  $^{32}\text{P}$  into an acid labile, base stable linkage characteristic of phosphohistidine. About 1.0 phosphate molecules per 140,000 M.W. could be incorporated from ATP- $\gamma$ - $^{32}\text{P}$  while somewhat higher levels of incorporation (up to 1.8 molecules per 140,000 M.W.) could apparently be obtained using  $^{32}\text{P}_i$  and succinyl CoA. It was also noted that a very low level of ATPase activity was associated with even homogeneous preparations of SCS and appeared to be a property of this enzyme. However the turnover of the ATPase activity is considerably faster than that of the phosphorylated derivative of SCS under similar conditions; thus the ATPase activity does not appear to be due to the hydrolysis of the phosphohistidine residue (13).

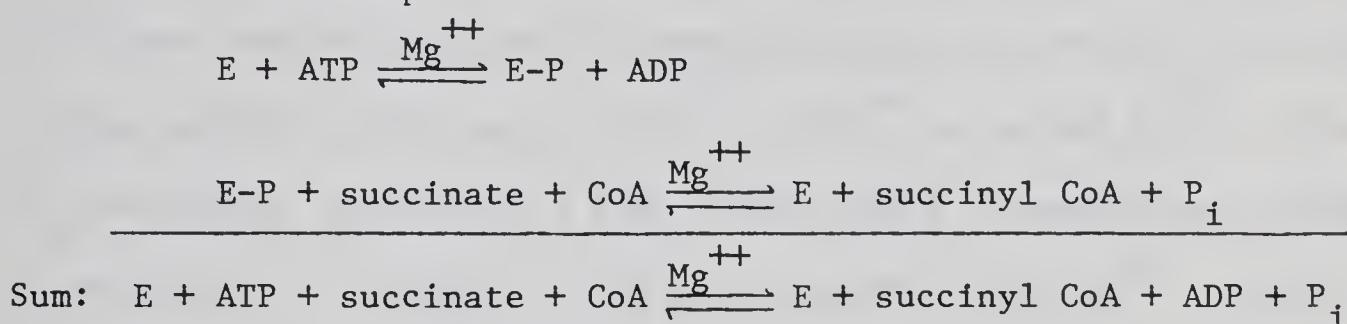
Bridger et al. (14) have demonstrated that the phosphohistidine residue formed by SCS meets kinetic requirements for a catalytic intermediate in the overall reaction. They showed that the pre-steady state rate of E-P formation in either direction was at least as great as the steady state rate of product formation in the same direction.

The above studies represent the first detection and characterization of phosphohistidine in a biological system. Since that time phosphohistidine residues have been detected or indicated in



several other protein systems including nucleoside diphosphate kinase (15), ATP-citrate lyase (16), glucose-6-phosphatase (17), phospho-glyceromutase (18) and in a bacterial sugar transport protein (19). However E. coli succinyl CoA synthetase remains the only enzyme for which the catalytic role of the phosphohistidine intermediate has been definitely established.

With the knowledge that a covalent enzyme-phosphate intermediate participates in the catalysis it is possible to write the overall reaction in two steps:

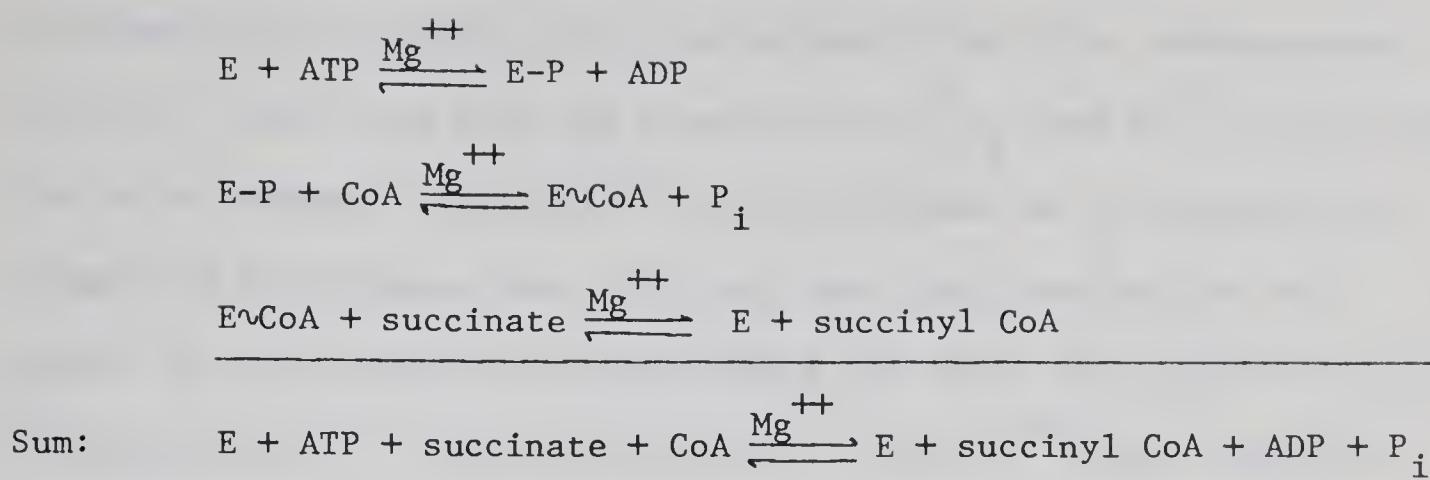


SCHEME I

Two distinct proposals were originally advanced in the literature concerning the mechanism of the second step of this scheme. One centered around participation of a high energy enzyme-bound CoA intermediate; the other, around a succinyl-phosphate intermediate.

The high energy  $E\sim\text{CoA}$  intermediate was proposed by Upper (5) and by Moyer et al. (20) for the E. coli enzyme and by Cha et al. (11) for the pig heart enzyme. The reaction sequence for such a mechanism could be written:





## SCHEME II

The proposal was based mainly on the observation that  $E-^{32}P$  was dephosphorylated by incubation with CoA and that the resulting enzyme species could react with  $^{32}P_i + Mg^{++}$  to reform  $E-^{32}P$ . This form of the enzyme, apparently a high energy  $E\sim CoA$  intermediate, could also be obtained by treatment of SCS with succinyl CoA +  $Mg^{++}$ . The CoA dependence of  $ATP \leftrightarrow P_i$  and  $E-P \leftrightarrow P_i$  exchange, as well as the apparent  $P_i$  independence of the succinate  $\leftrightarrow$  succinyl CoA exchange were also taken to be evidence in support of this mechanism.

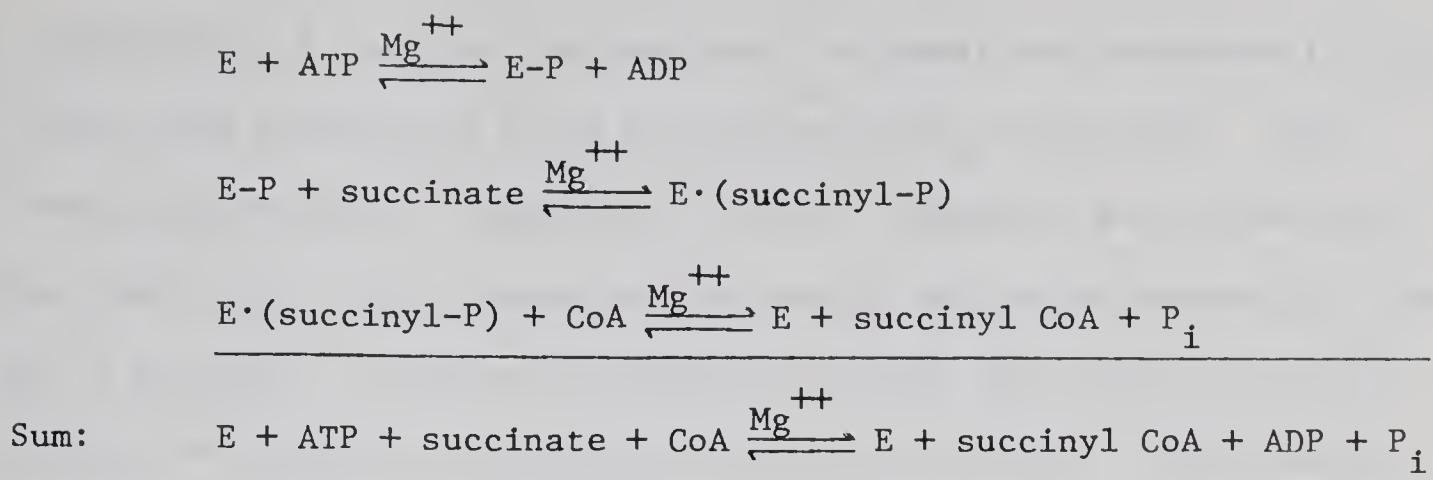
However it has been demonstrated (3) that oxygen is transferred from  $P_i$  to succinate in the course of the SCS reaction. If the mechanism involving  $E\sim CoA$  is correct the oxygen must be transferred through a group on the enzyme or on the CoA molecule. For example, Benson and Boyer (22) have established that such a transfer of oxygen through a group on the enzyme takes place in the succinyl CoA-acetoacetate transferase reaction which Hersch and Jencks (23) have shown to involve an enzyme-bound CoA species. Unfortunately in the case of succinyl CoA synthetase no such transfer could be demonstrated (20,24). In the light of this discovery Boyer's group



re-examined the evidence for the existence of an E $\sim$ CoA intermediate (24,25). They found that the liberation of  $^{32}P_i$  from E- $^{32}P$  by CoA was due to an exchange between E- $^{32}P$  and  $P_i$  (present as a contaminant in commercial CoA preparations (24)) and that this reaction did not result in the formation of enzyme-bound CoA (25). The capacity of SCS, dephosphorylated in such a reaction, to reform E- $^{32}P$  when incubated with  $^{32}P + Mg^{++}$  (the standard assay for E $\sim$ CoA) could be accounted for by exchange of  $^{32}P_i$  with E-P. They further found (24) that this enzyme shows a tendency for strong non-covalent binding of succinyl CoA which would account for the apparent formation of E $\sim$ CoA from SCS + succinyl CoA. Also, presence of only a trace of  $P_i$  contaminant during the succinyl CoA treatment would result in formation of E-P which could then exchange with  $^{32}P_i$  in the assay for E $\sim$ CoA. As well, evidence was obtained that commercial CoA contains traces of ATP or an ATP-like material which could phosphorylate SCS when excess amounts of a CoA preparation were added to the enzyme (26). It was also shown (25) that  $P_i$  would stimulate the succinyl CoA  $\longleftrightarrow$  succinate exchange and it appeared likely that this reaction was in fact phosphate-dependent.

The above results effectively rule out the participation of an enzyme-bound CoA intermediate in the SCS reaction. The default of this proposal lends support to the possible participation of an enzyme-bound succinyl phosphate intermediate. None of the above studies exclude such an intermediate and it accounts particularly well for the oxygen transfer between  $P_i$  and succinate and for a  $P_i$  dependent succinate  $\longleftrightarrow$  succinyl CoA exchange, as can be seen from the following scheme:





## SCHEME III

The possibility of an enzyme-bound succinyl phosphate was originally considered by Hager (3) in 1962 but there was little evidence available at that time to support the concept. In recent years considerable evidence has accumulated in favour of such an intermediate. The enzyme was shown by Nishimura and Meister (27) to react with succinyl phosphate and CoA to give succinyl CoA and with succinyl phosphate and ADP to give ATP. They also detected succinyl-P synthesis by SCS in the presence of ATP and succinate, and showed that succinyl-<sup>32</sup>P could phosphorylate the enzyme. Subsequently Nishimura (28) demonstrated that E-<sup>32</sup>P would react with succinate to produce succinyl-P which dissociated from the enzyme in the absence of CoA. In the presence of CoA and succinate the reaction rapidly produced P<sub>i</sub> + succinyl CoA and no succinyl-P could be detected.

The major objection to the participation of succinyl-P as an intermediate in the reaction is based on kinetic considerations. All of the above reactions involving succinyl-P take place at a very slow rate relative to the overall reaction (25,27,28) and require high substrate concentrations. It was noted (25,28) that in the presence of CoA the formation of an enzyme-bound succinyl-P might be rapid enough

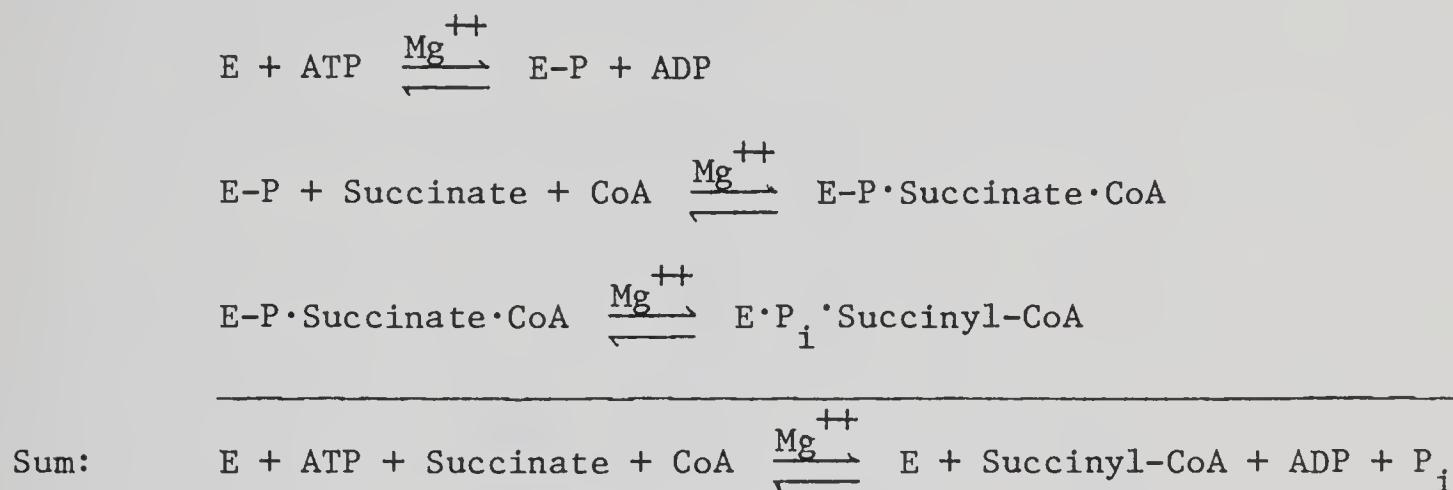


to qualify as a reaction intermediate. Grinnell and Nishimura (29,30) tested this possibility using the CoA analogue, desulfo-CoA. They found that succinyl-P synthesis from ATP + succinate was stimulated, but that very little stimulation occurred in succinyl-P production from E-P + succinate. Further investigation showed that ATP could inhibit succinyl-P formation at relatively low concentrations (approximately 0.1 to 0.2 mM) (29), and that desulfo-CoA would antagonize this inhibition. Hildebrand and Spector (31) have demonstrated that desulfo-CoA will markedly stimulate ATP- $\gamma$ -<sup>32</sup>P production from succinyl-<sup>32</sup>P, but did not examine the formation of E-<sup>32</sup>P. Despite the observed stimulatory effects, the best rates of succinyl-P utilization in the presence of desulfo-CoA were five-fold slower than the rates of the overall reaction (31).

These same authors have reported that succinyl-P will react rapidly and nonenzymically with CoA to produce succinyl CoA (31). In subsequent work they found that various dicarboxylic acid phosphates would react with CoA in a similar manner (32). Further, they have obtained evidence that succinyl-P, a cyclic form of succinyl-P, and succinic anhydride exist in equilibrium in aqueous solution. It appears that at neutral pH CoA reacts with the cyclic form of succinyl-P to produce succinyl CoA (32). These findings provide at least intuitive support for the participation of succinyl-P as an intermediate in the SCS reaction, since its chemical properties would appear to be functional in this system.



Despite the above evidence that it can function in various stages of the overall reaction, succinyl-P has still not been shown to meet kinetic criteria for an intermediate in catalysis. This fact has led Robinson et al. (25) to reconsider a concerted reaction mechanism similar to that proposed by Hartman and Buchanan (33,34) for several ATP linked synthetases catalyzing formation of amine or amide bonds. Such a mechanism in relation to catalysis by SCS was earlier considered by Hager (3). This mechanism as applied to the SCS reaction would be written:



SCHEME IV

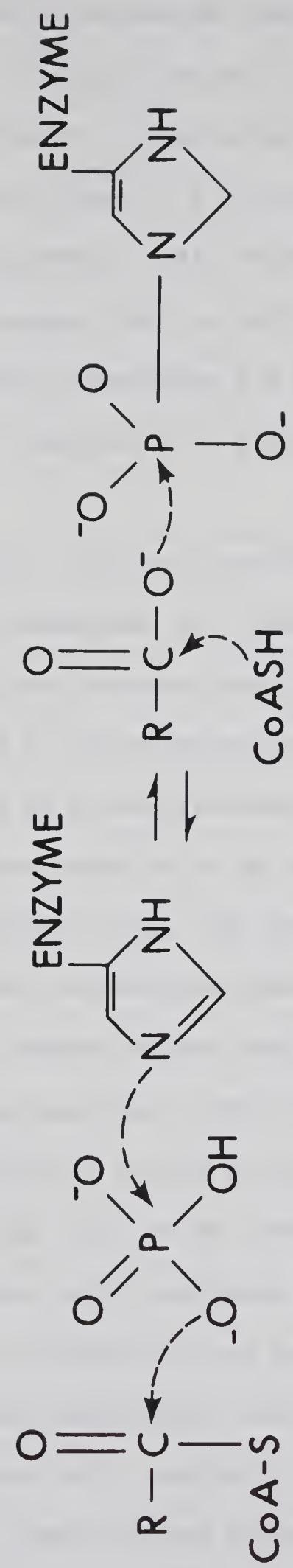
Figure 1 shows the molecular mechanism of such a reaction as proposed by Robinson et al. (25).

This mechanism does not involve succinyl-P as a discrete intermediate in the catalysis. Robinson et al. (25) rightly point out that such a mechanism is difficult to test but it gains support from the lack of a kinetically demonstrable catalytic intermediate for this phase of the reaction.





Figure 1. Proposed molecular mechanism of a concerted reaction for SCS (Robinson *et al.* (25)).





Such a concerted mechanism involving all substrates in a central complex can be rationalized with exchange reactions not requiring all substrates by consideration of the fact that these partial reactions in the absence of other substrates are much slower than they are in the presence of all substrates (12,14,21,35). Robinson et al. (25) suggest that an active center containing subsites to align several substrates for a concerted reaction might well catalyze partial reactions at a much reduced rate in the absence of some substrates.

Bridger et al. (14) have considered this phenomenon, which they called substrate synergism, on a theoretical as well as experimental basis. These workers using the approach developed by Boyer (36,37) obtained a rate equation for isotope exchange between one substrate-product pair of a multireactant system in the presence of all reactants. The rate was found to be an inverse function of the concentration of substrates other than the exchanging pair. This result follows from the consideration that these other reactants will combine with the enzyme to form complexes which are not active in the exchange reaction. With these predictions in mind several exchange reactions catalyzed by SCS were examined. The rate of ATP  $\longleftrightarrow$  ADP exchange was found to be increased five-fold in the presence of all other substrates, while succinate, CoA, and P<sub>i</sub> added separately gave slight stimulation and succinyl CoA gave marked stimulation. They also demonstrated that CoA would stimulate the exchange between E-P and ATP. Succinate  $\longleftrightarrow$  succinyl CoA exchange was found to be 120 times faster in the presence of all substrates than in



their absence. Individual substrates  $P_i$ , CoA, and ADP gave slight stimulation while ATP gave marked stimulation. All of these results are opposite to the theoretical rate predictions described above and provide clear evidence that some type of synergistic interaction takes place in this mechanism when all substrates are present. In other words it would appear that all subsites at the active center must be occupied in order to achieve the most active configuration for catalysis of partial reactions.

All of the mechanism proposals detailed earlier include or imply dissociation of ADP from the enzyme after formation of the E-P intermediate. Such a step is not consistent with the observation (above) that all substrates including adenine nucleotides are required for maximal rate of succinate  $\rightleftharpoons$  succinyl CoA exchange, nor is it consistent with the stimulation of ATP  $\rightleftharpoons$  ADP exchange by the addition of succinyl CoA. Scheme IV, the concerted mechanism can be brought into line with the data by assuming that ADP remains bound to the enzyme and exerts some effect on the catalysis (i.e. substrate synergism). Scheme III will also be in harmony with the exchange results if it is assumed that formation and hydrolysis of the succinyl-P intermediate occurs in a central complex involving all substrates. The phenomenon of substrate synergism thus suggests definite mechanistic possibilities for the SCS reaction and it is with these possibilities that this dissertation will be concerned.

Since SCS is known to form a covalent enzyme-substrate intermediate, it might be expected to show ping-pong initial rate kinetic patterns (38) as are generally seen for enzyme reactions



involving such intermediates (39,40). However the substrate synergism observations with this enzyme suggest that despite the presence of a E-P covalent intermediate, all reactants are involved in the central complex and thus a sequential rather than ping-pong kinetic mechanism should be observed. This dissertation presents evidence that in fact a partially random sequential mechanism is the major catalytic pathway of the SCS reaction. Kinetic studies of ATP  $\rightleftharpoons$  ADP isotope exchange at equilibrium indicate that minor pathways are also allowed and that a fully random sequential kinetic mechanism is probably operative.

The stimulatory effect of substrates on partial reactions observed with SCS may be mediated through substrate induced conformational changes in the protein molecule. As well, structural studies on SCS have led to a problem in the correlation of the number of phosphate groups incorporated with the number of subunits apparently present. The dissociation of SCS into subunits was first reported by Ramaley et al. (12) and later by Grinnell et al. (41) but no characterization of the products was reported. Leitzmann et al. (42) have reported a more detailed examination of dissociation conditions including use of p-mercuribenzoate, sodium dodecyl sulphate, guanidine hydrochloride, iodoacetamide, and succinic anhydride. They obtained evidence for four subunits of similar identical molecular weight, about 40,000, but aggregation occurred in all systems making the results somewhat uncertain. Recently, Bridger (43) has obtained clear evidence that SCS will dissociate into two types of subunits, separable by SDS-polyacrylamide gel electrophoresis. He obtained molecular weights



of 29,500 and 38,500 for these subunits, strongly suggesting that the native enzyme is a tetramer of an  $\alpha_2\beta_2$  type of composition. Furthermore it was found that only the smaller subunit is phosphorylated in the SCS reaction. Ramaley et al. (12) reported that SCS will usually incorporate about 1.0 to 1.4 phosphate molecules per 140,000 M.W. depending on conditions. Leitzmann et al. (42) repeated this result, but Grinnell et al. (35) obtained the somewhat higher value of approximately 1.8 phosphate molecules per 140,000 M.W. In general the enzyme is seen to incorporate one phosphate group per molecule with ease from either direction, but further phosphorylation occurs with some difficulty. These results may be rationalized with the subunit structure suggested by Bridger by considering the possibility of a conformational change on phosphorylation resulting in an anti-co-operative effect of the type recently reported by Levitzki et al. (44). This dissertation reports evidence, obtained from trypsin-catalyzed inactivation studies, that a conformational change does occur upon phosphorylation. This conformational change was seen to have a profound effect on the stability of the enzyme in solution as well as providing protection from trypsin-catalyzed inactivation.

Some preliminary studies of succinate binding to the phosphorylated enzyme and ADP binding to dephosphorylated enzyme are also reported.



## CHAPTER II

## EXPERIMENTAL PROCEDURE

A. Preparation of Materials

Succinyl CoA synthetase was prepared from succinate-grown E. coli (Crooke's strain) according to the method of Leitzmann et al. (42) with the following modifications; QAE-Sephadex was used in place of DEAE-Sephadex for ion exchange chromatography and a final chromatographic step on G-100 Sephadex was added. The purification results in enzyme that is chromatographically and electrophoretically homogeneous. The apparently homogeneous enzyme preparations exhibit a range of specific activities from about 20 units per milligram to a maximum of greater than 40 units per milligram. Except where otherwise noted, enzyme used in the experiments reported here had a specific activity greater than 20 units per milligram.

SCS as isolated was stable when stored as the precipitate, in 75% saturated  $(\text{NH}_4)_2\text{SO}_4$  at 4°C. In solution the dephosphorylated form of the enzyme was highly unstable in the absence of phosphate with a half-life of about 24 hours in 50 mM Tris-Cl, 50 mM KCl, 0.1 mM EDTA at pH 7.2. The stability of the enzyme and its phosphorylated form is considered more fully in Chapter V. Routinely the enzyme was stored as the precipitate in 75%  $(\text{NH}_4)_2\text{SO}_4$  and small amounts were prepared for use by centrifugation, solution in 50 mM Tris-Cl, 50 mM KCl, 0.1 mM EDTA, pH 7.2, followed by chromatography on a 50 cm x 1 cm G-50 Sephadex column equilibrated in the same buffer.



Dephosphorylated SCS was prepared by incubation of the enzyme with ADP + Mg<sup>++</sup> in the presence of a glucose-hexokinase trap followed by chromatography on G-50 Sephadex equilibrated with Tris-Cl buffer as described above. E-<sup>32</sup>P was prepared by incubation of SCS with excess ATP-γ-<sup>32</sup>P + Mg<sup>++</sup> in 50 mM Tris-HCl, 50 mM KCl, pH 7.2 for 15 minutes at 25°C, followed by chromatography on G-50 Sephadex also as described above. E-<sup>32</sup>P was assayed by the phenol extraction technique as described by Ramaley et al. (12).

Chemicals were of the highest grade commercially available and, except where noted, were used without further purification or treatment. CoA and succinyl CoA were purchased from P.L. Biochemicals. Hexokinase was obtained from Sigma Chemical Company and TPCK-trypsin (trypsin treated with TPCK to inactivate chymotrypsin) was the product of the Worthington Biochemical Corporation. β,γ-methylene-ATP (Aopopcp) was purchased from Miles Laboratories.

ADP-(G)-<sup>3</sup>H, succinyl-2,3-<sup>3</sup>H acid and [<sup>32</sup>P]-orthophosphate were obtained from New England Nuclear. ATP-<sup>14</sup>C(U) was the product of Amersham/Searle. ADP-<sup>14</sup>C (U) was prepared by the method of Bridger et al. (14). The ADP-<sup>14</sup>C so prepared was found to be better than 97% radiochemically pure on thin layer chromatography as described below. ATP-γ-<sup>32</sup>P was prepared from [<sup>32</sup>P]-orthophosphate by the method of Ramaley et al. (12).

Pre-coated plastic cellulose PEI-F thin layer chromatography sheets were obtained from the J.T. Baker Chemical Company. They were prepared for use essentially as described by Randerath and Randerath (45).



Cells for rate of dialysis binding studies were prepared by modification of Chemical Rubber Company 1 ml constant flow dialysis cells as described by Colowick and Womack (46). Equilibrium dialysis cells of 0.1 ml capacity were supplied by Bel-Art products. Dialysis membrane sheets were also obtained from Bel-Art and were prepared by boiling in 0.1 M EDTA for 15 minutes, followed by rinsing in distilled water. The membranes were stored in distilled water and were equilibrated in 50 mM Tris-HCl, 50 mM KC1 buffer at pH 7.2 before use.

#### B. Initial Velocity Assays

Routine assays of SCS were carried out essentially as described by Ramaley et al. (12) using the increase in absorption at 230 nm which accompanies the formation of succinyl CoA. The assay mixture contained 10 mM succinate, 100  $\mu$ M CoA, 0.38 mM ATP, 10 mM MgCl<sub>2</sub>, 50 mM Tris-Cl, pH 7.2 in a 1 ml volume in a 1 cm path length cuvette. The appearance of succinyl CoA was followed at 230 nm in a Cary model 15 recording spectrophotometer. A similar assay can be used in the reverse direction. The assay mixture in this case contains 50 mM P<sub>i</sub>, 100  $\mu$ M succinyl CoA, 0.38 mM ADP, 10 mM MgCl<sub>2</sub>, 50 mM Tris-Cl, pH 7.2 and the disappearance of succinyl CoA at 230 nm is followed. This assay is found to give a value of about 90% of the velocity in the succinyl CoA formation direction for the same amount of enzyme. All routine assays were carried out in the forward direction, and one unit of SCS is always defined as the amount of enzyme catalyzing the formation of one  $\mu$ mole of succinyl CoA per minute at 25°C. Velocity vs concentration of enzyme plots were uniformly linear to enzyme concentration 100 times those used in experimental kinetic assays.



In the experimental initial velocity assays it was necessary to measure the rate of very small changes in absorbance at 230 nm with substantial blanks in order to obtain accurate kinetic data. Accordingly kinetic measurements were performed on the Cary Model 15 with 0 to 0.1 absorbance slidewire and, except where indicated, cells with a 4 cm light path and a working volume of 3 ml. Under these conditions, even at low substrate concentration, the reaction was linear for several minutes and initial rates were readily estimable. Rates are expressed as micromoles of succinyl CoA formed (or cleaved) per minute per ml at 25°C, using the value of 4.5 per cm for the increase in the millimolar extinction coefficient at 230 nm (47). The concentration of  $MgCl_2$  was held at 10 mM throughout. This is an optimal concentration even at the lowest substrate concentrations used, and we have made the simplifying assumption that this concentration is sufficiently high that kinetic effects of  $Mg^{++}$  need not be considered.

Kinetic data, which appeared to give linear reciprocal plots were analyzed with the aid of a programme written for the Olivetti-Underwood Programma 101 which performs a weighted least squares fit according to the method of Wilkinson (48). The program also furnishes the standard error of the apparent  $K_m$  and  $V_{max}$  for each set of data.

### C. Isotope Exchange Assays

The equilibrium constant for succinyl CoA synthetase in the direction of succinyl CoA formation has been determined to be approximately 0.27 (49). In experiments to test the effect of raising the concentration of a substrate on the exchange rate, reaction mixtures were set up to approximate this equilibrium for each substrate concentration. The



varied substrate was changed in constant ratio with a product to maintain the equilibrium. Preliminary experiments were conducted to establish enzyme and ADP-C<sup>14</sup> concentrations as well as appropriate sampling times for each experiment.

After addition of enzyme the reactions were incubated at 25°C for 90 minutes to allow for minor adjustments to chemical equilibrium. About 250,000 cpm of ADP-C<sup>14</sup> (531 mc/mM) was added to each reaction. The very small amount of ADP added as ADP-C<sup>14</sup> was assumed to have a negligible effect on the chemical equilibrium. 50 µl samples were taken from each reaction at times before and after isotopic equilibrium as determined in preliminary experiments and added to 25 µl of 0.1 M EDTA in test tubes which were then placed in an ice bath. 10 µl of each of these samples was spotted on a 20 x 20 cm PEI-F cellulose thin-layer chromatography sheet prepared as described above. The plates were run to a height of about 18 cm in 1.0 M LiCl and the ADP and ATP spots were identified under ultraviolet light. The spots were cut out of the plastic plates and counted directly in a scintillation vial with 15 ml of a toluene based scintillation fluid (50) with the use of the Nuclear Chicago Mark I liquid scintillation system. Enzyme blanks containing all substrates and ADP-C<sup>14</sup> were run for each experiment and all counts were corrected for these blanks.

Rates of isotope exchange were calculated from the equation

$$R = \frac{[ADP] \times [ATP]}{[ADP] + [ATP]} \times \frac{1}{t} \times \ln (1-F)$$

as discussed by Boyer (36,37). [ATP], [ADP] are the equilibrium concentrations of these substrates calculated from the total initial



concentration of adenine nucleotide and the  $^{14}\text{C}$  distribution at isotopic equilibrium. F is the fraction of isotopic equilibrium at time t. Rates were calculated for samples taken at times before 50% of isotopic equilibrium is attained and are reported as the average  $\pm$  standard deviation for three such samples from each reaction mixture. Rates are given in units of  $\mu\text{moles per minute per ml}$  at  $25^\circ\text{C}$ .

#### D. Trypsin-Catalyzed Inactivation Assays

Activity assays were carried out using the standard spectrophotometric assay described earlier under initial rate kinetic assays. Reaction mixtures were incubated at  $25^\circ\text{C}$  and samples at appropriate times were pipeted directly into 1.0 ml cuvettes containing complete assay mixtures in the Cary Model 15 spectrophotometer. Zero time activities were determined before the reactions were initiated by the addition of trypsin. Activities were determined as units per ml of the original reaction mixture. Protein bound phosphohistidine was determined by the phenol extraction method described by Ramaley *et al.* (12).

Results were calculated as percentages of original activity remaining at time t and are plotted according to the first order equation

$$\ln \frac{[E_0]}{[E]} = kt$$

where  $[E_0]$  and  $[E]$  are the activity concentrations at times  $t_0$  and t respectively, and k is the pseudo-first order rate constant for the inactivation process. k values were calculated from data which appeared to be linear in these plots from a regression analysis using a programme written for the Olivetti-Underwood programma 101.



#### E. Substrate Binding Studies

Studies of the binding of  $^3\text{H}$ -ADP to SCS were carried out by the rate of dialysis technique described by Colowick and Womack (46). The dialysis cell had a lower chamber volume of 2.8 mls and an upper chamber volume of about 2.5 mls. In practice a 1.0 ml volume of enzyme solution plus radioactive substrate is added to the upper chamber initially, followed by successive addition of unlabelled substrate at appropriate times. Flow rate of buffer (50 mM Tris-Cl, 50 mM KCl, pH 7.2) through the lower chamber was 3.3 ml per minute. Fractions of 3 ml volume were collected and 1 ml aliquots of each fraction were counted in 15 mls of Bray's solution (51). Successive substrate additions were made at 8 fraction intervals, allowing a new steady state level of radioactivity in the lower chamber to be reached between each substrate addition (46). Data were analyzed as described by Colowick and Womack (46) and are plotted according to the method of Scatchard (52).

Equilibrium dialysis of  $^3\text{H}$ -succinate binding to the phosphorylated enzyme was carried out in cells which had a total capacity of 0.2 ml divided into two chambers by dialysis membrane treated as described above. Enzyme was introduced into one compartment and ligand into the other with the use of a Hamilton 100  $\mu\text{l}$  syringe and a teflon needle. Both sides contained 50 mM Tris-Cl, 50 mM KCl, (pH 7.2) and 10 mM EDTA. Total ligand concentration was varied from cell to cell, but the total amount of radioactivity added to each cell was constant at  $1.5 \times 10^4$  cpm per cell. The time required for equilibration was found to be approximately 4 hours in preliminary experiments. The cells



were placed in a horizontal shaker at room temperature for equilibration. Triplicate 10  $\mu\text{l}$  samples were withdrawn from each chamber with disposable capillary pipets after equilibration. The samples were pipetted into 1 ml of distilled water in scintillation vials. 15 mls of Bray's solution was added and the vials were counted in the Nuclear Chicago Mark I liquid scintillation system. Samples were also withdrawn from the protein compartment after equilibration for determination of protein concentration and enzyme activity. The activity of the phosphoenzyme was found to be stable under these conditions and protein concentrations remained unchanged over the course of an experiment. Data were analyzed with the aid of the following equations:

$$C_f = \frac{C_I D_f}{D_p + D_f}; \quad C_B = \frac{C_I (D_p - D_F)}{D_p + D_F}$$

where  $C_f$  and  $C_B$  are the concentrations of free and bound ligand respectively.  $C_I$  is the initial concentration of ligand in one compartment and  $D_p$  and  $D_f$  are cpm in samples from compartments containing and lacking protein respectively (54). Data were again plotted by the Scatchard method and slopes and intercepts were obtained from the regression analysis programme referred to above.

Enzyme for binding experiments was collected by centrifugation from 75%  $(\text{NH}_4)_2\text{SO}_4$  and was taken up in a small volume of 50 mM Tris-Cl, 50 mM KCl, pH 7.2. The enzyme was concentrated and any  $(\text{NH}_4)_2\text{SO}_4$  removed by diafiltration with this buffer using an Amicon micro-ultrafiltration system, Model 8MC with a UM-10 membrane. Phosphorylated SCS was prepared as described previously, but in place of chromatography on G-50 Sephadex, the reaction mixture was placed in the ultrafiltration



apparatus, concentrated to about 1 ml and diafiltered with 100 mls of 50 mM Tris-Cl, 50 mM KCl, pH 7.2, to remove substrates. The same technique could be used for preparation of dephosphorylated SCS; however the instability of this form of the enzyme results in appreciable loss of activity during the filtration process.

Fluorescent spectra were obtained using a Turner Model 210 fluorescent spectrophotometer. In a typical experiment an emission spectra for free enzyme using excitation at 280 nm was run on a solution of 0.2 mgm of SCS in 2 ml of 50 mM Tris-Cl, pH 7.2 at 25°C. Ligands were added to the enzyme solution directly in the cuvette and the emission spectra superimposed on the free enzyme spectrum. Changes observed in emission peak heights in the presence of ligand were corrected for volume changes due to ligand addition.

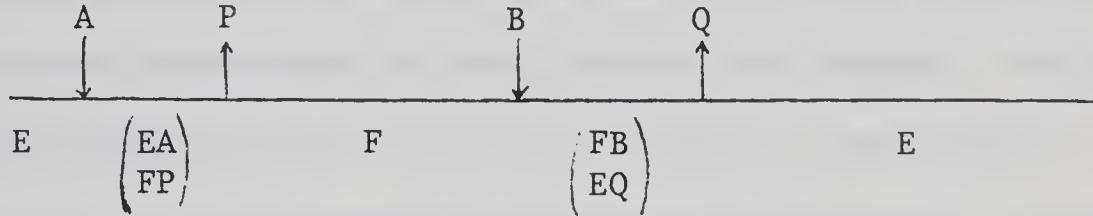


## CHAPTER III

## KINETIC MECHANISM OF SUCCINYL CoA SYNTHETASE. INITIAL VELOCITY STUDIES

A. Introduction

Enzymes which form covalent enzyme-substrate intermediates often display the type of kinetic mechanism which Cleland (38) has called "ping-pong". Such a mechanism involves the release of at least one product from the enzyme before all substrates have been added. As a result, the enzyme will oscillate between two distinct forms. Consider as an example the two-substrate mechanism that would be designated bi-bi ping-pong in Cleland's nomenclature (38):



In this case product P dissociates to produce a stable form of enzyme F, which is a complex of E and some group from A. F then reacts with B to form Q and reform E. The enzyme will thus oscillate between the two stable forms E and F. Examples of this mechanism include glutamic oxaloacetic transaminase (40) where E is the pyridoxal form of the enzyme and F the pyridoxamine form, and nucleoside diphosphate kinase (39,54) where E and F are respectively the free enzyme and a phosphorylated form of the enzyme.

Cleland's rules for the prediction of initial velocity patterns by inspection (38) state that parallel double reciprocal plots will be observed when there is an irreversible step between the addition of variable and fixed variable substrates in an initial velocity

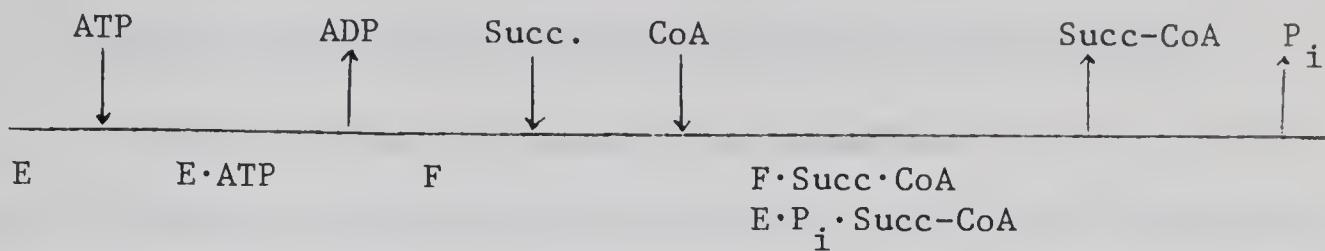


experiment. The release of a product under initial velocity conditions will be such an irreversible step, and since ping-pong mechanisms involve alternate substrate addition and product release, parallel double reciprocal plots of initial velocities will be characteristic of such mechanisms.

A ping-pong mechanism, then, implies the formation of an intermediate between free enzyme and a substrate group to form a second stable enzyme species (designated F in the above discussion). Enzymes which form covalent enzyme-substrate intermediates commonly display ping-pong kinetic mechanisms (39,40,54) where the covalent enzyme-substrate corresponds to the F form of the enzyme. One cannot, however, regard ping-pong kinetics as an invariant characteristic of mechanism involving covalent enzyme-substrate intermediates. Initial rate kinetics can give information only about the order of substrate addition and product release. Thus ping-pong kinetics will be observed for enzymes with covalent intermediates only if a product does, in fact, dissociate after the formation of the intermediate and before subsequent substrate addition. This distinction is important because the widespread observation of ping-pong kinetics associated with the occurrence of covalent enzyme-substrate intermediates may give rise to a tendency to consider the lack of such kinetics as conclusive evidence against the participation of such covalent intermediates in the reaction mechanism. Succinyl CoA synthetase provides an excellent demonstration of this point. This enzyme has been shown to involve a phosphorylated enzyme as a catalytic intermediate (14). A reasonable ping-pong kinetic mechanism could



be written for the reaction as follows:



where F corresponds to the covalent E-P intermediate. However, the observation of substrate synergism in association with this reaction (see CHAPTER I) suggests that the reaction involves a quaternary complex of all substrates implying a sequential mechanism despite the participation of the covalent intermediate. The initial rate kinetic studies on succinyl CoA synthetase presented in this Chapter support this possibility.

It should be noted that the term "ping-pong kinetics" referring to initial velocity double reciprocal plots that display parallel lines is somewhat restrictive when dealing with enzymes having three or more substrates. In this case the addition of the third substrate at saturating concentration will constitute an irreversible step in the reaction sequence. If this addition occurs between the binding of the variable and fixed variable substrate, parallel plots will arise even though the mechanism is sequential (55). These parallel plots would, however, become intersecting when the concentration of the third (fixed) substrate was less than saturating. This is in contrast to the case of a ping-pong mechanism in which the irreversible steps are product dissociations where plots will remain parallel at all concentrations of fixed substrate. The kinetic studies of SCS will also provide an illustration of this point.



## B. Results and Discussion

### 1. Initial Rate Kinetic Studies of Succinyl-CoA Formation

Figure 2 shows the result of an experiment in which ATP is varied at several concentrations of succinate, while the concentration of CoA is held fixed at approximately 1.5 times the  $K_m$  value (see below). The results are a set of nonparallel lines which may be drawn to a common point of intersection within the standard errors of the coefficients. Figure 3 shows replots of slope and intercept as a function of the reciprocal of the succinate concentration. The fact that the slopes of the primary plots are significantly increased by lowering the succinate concentration indicates that the dissociation of ADP from the enzymes does not precede the attachment of succinate as schemes I-IV (Chapter I) imply.

When the experiment was repeated, but with the concentration of CoA raised 5-fold, the kinetic pattern of figure 4 emerged. The primary plots are parallel with no significant change in slope as shown by the inset to figure 4. Also shown is the linear dependence of the intercepts of the primary plot upon the reciprocal of the succinate concentration. The same experiment was repeated with a further increase in the CoA concentration and again parallel double reciprocal plots clearly arose. This experiment is illustrated in figure 5. The inset to figure 5 demonstrates that the slopes were again constant and that intercepts showed a linear dependence when plotted against the reciprocal of the succinate concentration. In this case, the primary plots continue to move closer together, i.e. lower concentrations of succinate were required in order to obtain



suitable vertical displacements. Thus the experiments with ATP and succinate varied at both high and low fixed concentrations of CoA suggest that CoA can be the second substrate to add in a sequential addition of substrates to form a quaternary complex.

Further experiments with ATP and CoA varied at fixed succinate concentrations show that a simple ordered sequential mechanism does not suffice to describe the kinetic mechanism. Figure 6 shows the patterns obtained when ATP and CoA are varied at a concentration of succinate near  $K_m$ . These lines are drawn by computer but they can intersect at a common point within the standard errors of their coefficients. The inset to figure 6 shows that both the slope and intercept of the primary plots are linear functions of the reciprocal of the CoA concentration. When succinate is made 5-fold more concentrated, however, a parallel pattern results as shown in figure 7. The inset shows the slope to be constant at all CoA concentrations. At this higher succinate concentration, the intercept is less strong a function of the CoA concentration as was seen in the analogous case of figures 2 and 4. Thus CoA and succinate appear to be kinetically equivalent since a high concentration of either produces parallel lines when the other two substrates are varied.

A model which appears to be largely consistent with the above data is MECHANISM I, which is depicted in figure 8. Such a model predicts the observed equivalence of CoA and succinate in the experiments reported above; each can be the second substrate to add to the enzyme when present near saturating concentrations. A rate equation may be derived for this mechanism with the use of the method of King and Altman (56):





Figure 2. Reciprocal plots showing effects of varying ATP and succinate at a fixed non-saturating concentration of CoA. The reaction mixtures contained, in a final volume of 3.15 ml, 0.1 M tris-HCl, pH 7.2, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 2.1  $\mu$ M CoA, SCS (0.003 units) and succinate as follows: o — o, 2.38 mM;  $\Delta$  —  $\Delta$ , 0.48 mM; • — •, 0.24 mM.

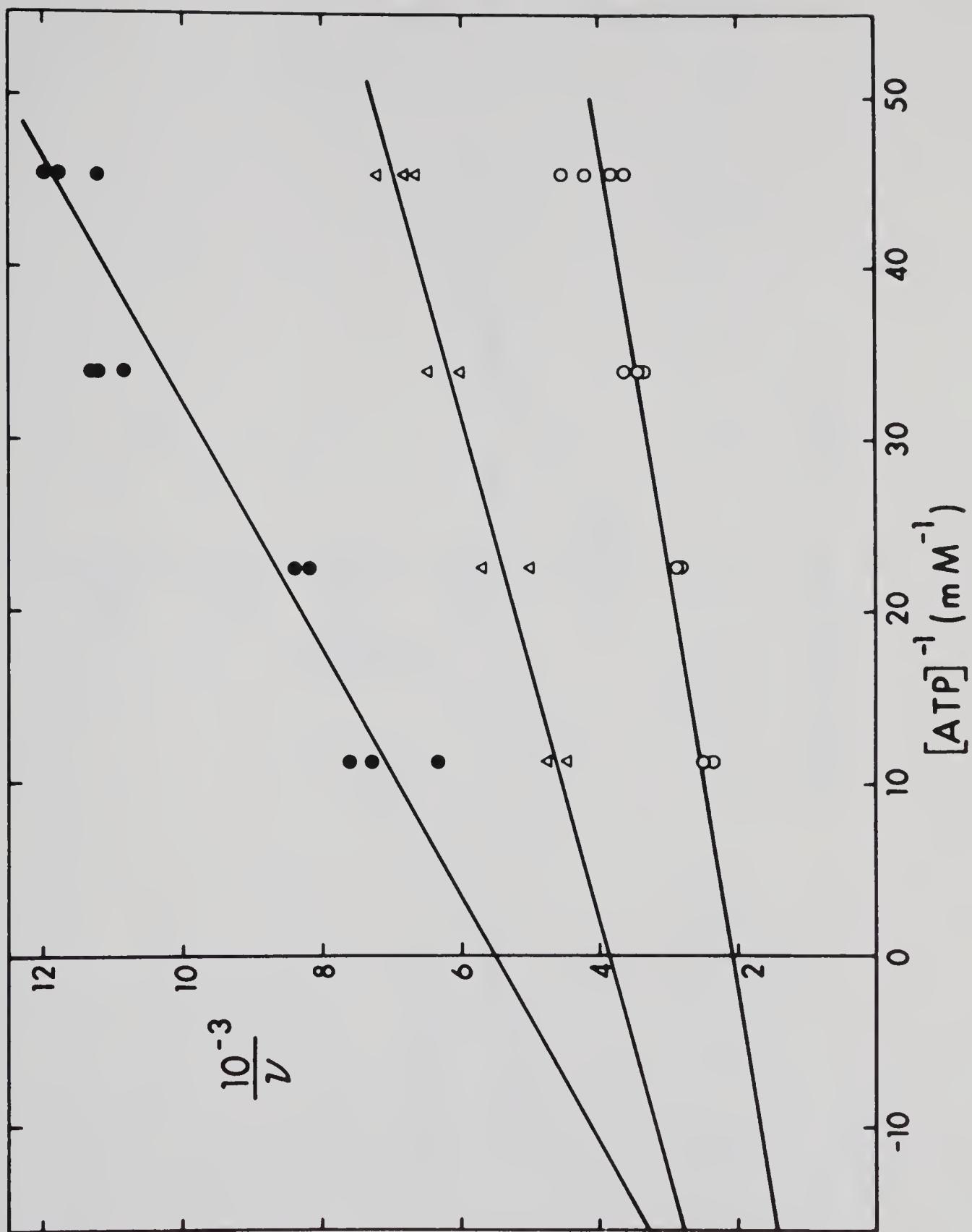






Figure 3. Replots of intercept (A) and slopes (B) of figure 2 versus reciprocal of the succinate concentration.

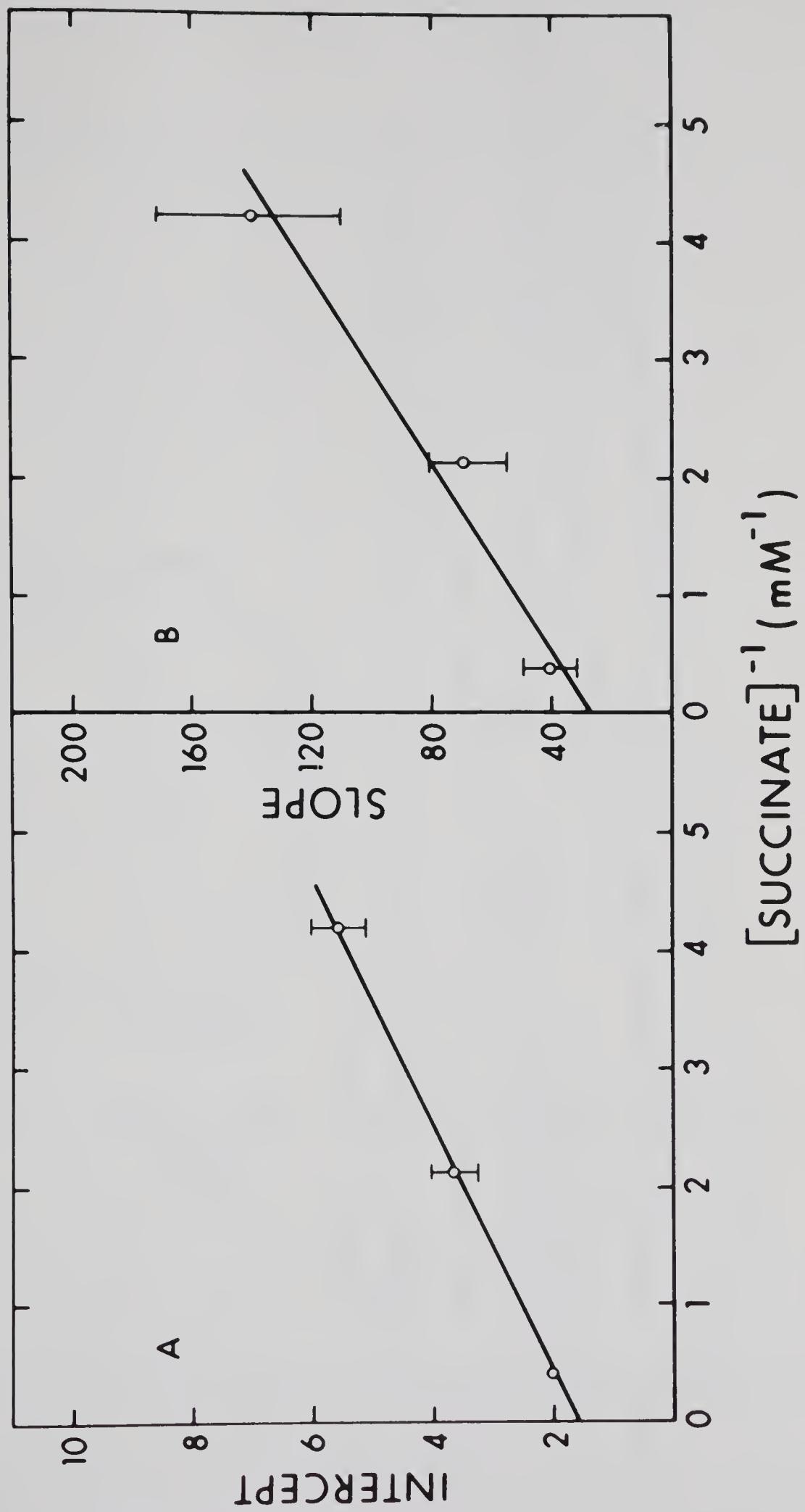






Figure 4. Reciprocal plots showing effects of varying ATP and succinate at a fixed, near-saturating concentration of CoA. The reaction mixtures contained, in a final volume of 3.15 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10.9 μM CoA, SCS (0.003 units) and succinate as follows: o —— o, 2.38 mM; Δ —— Δ, 0.24 mM; • —— •, 0.12 mM. The inset shows replots of slopes and intercepts as functions of the reciprocal of the succinate concentration.

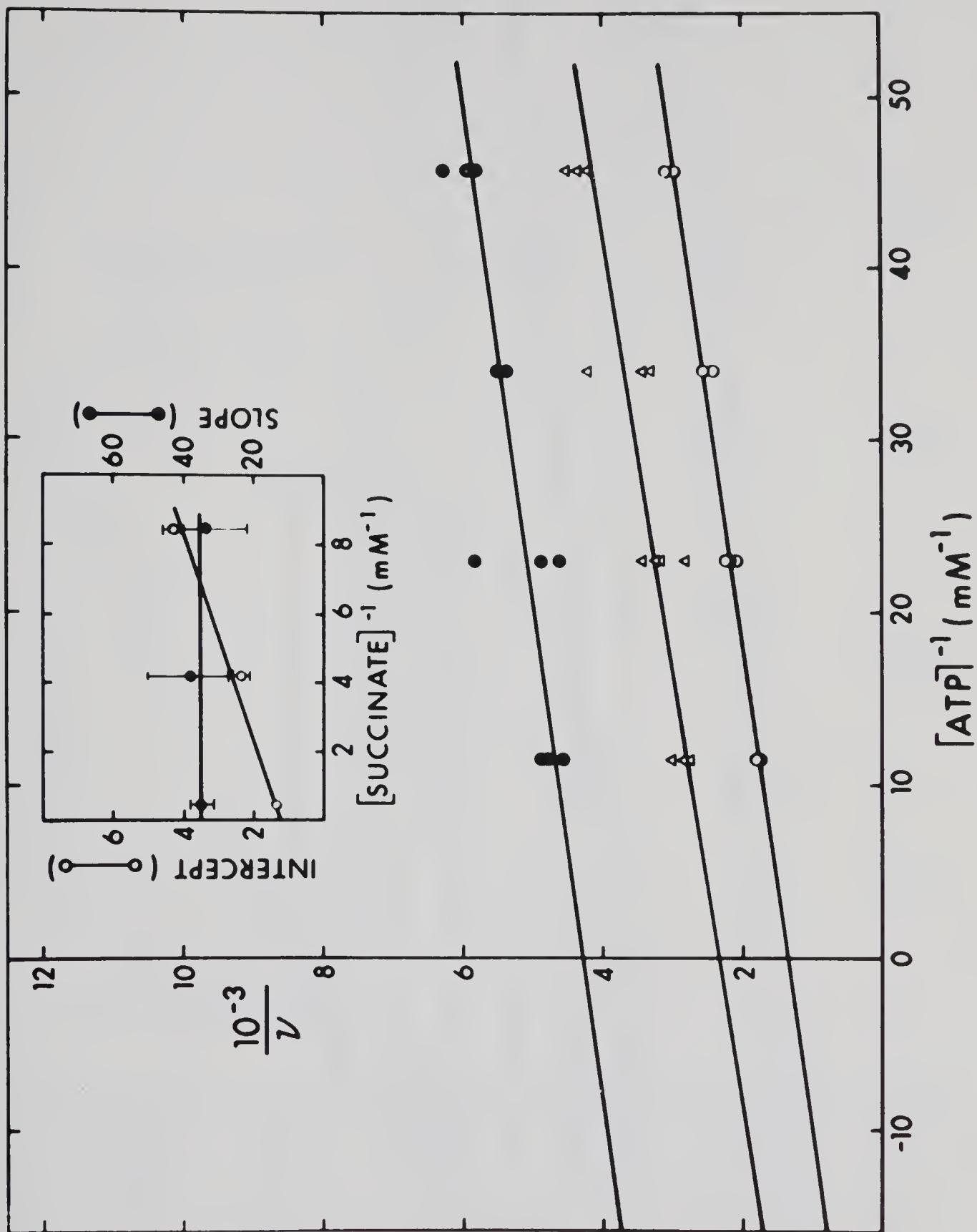
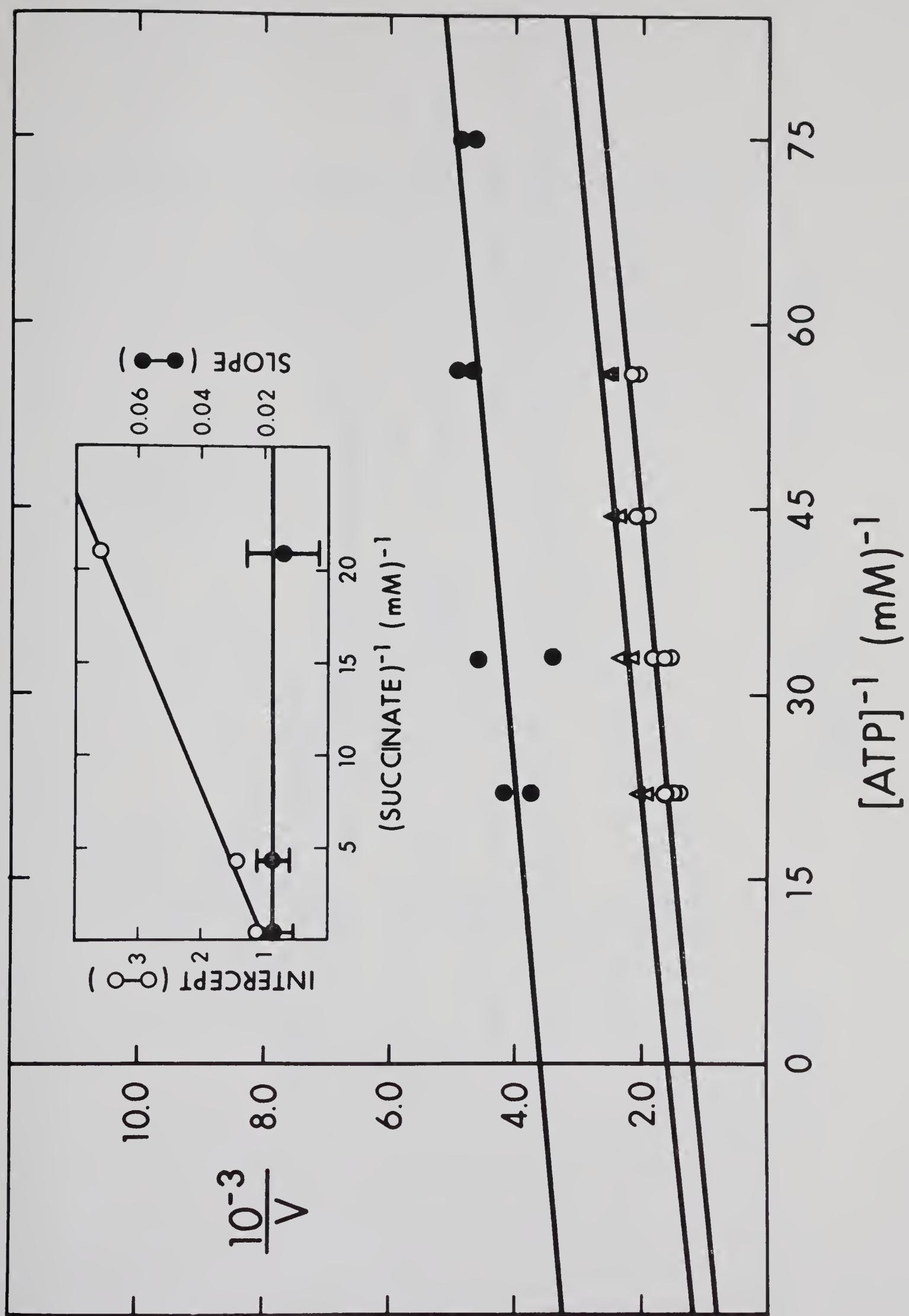






Figure 5. Reciprocal plots showing effects of varying ATP and succinate at a fixed, saturating concentration of CoA. The reaction mixtures contained in a final volume of 3.15 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 87 μM CoA, SCS (0.003 units) and succinate as follows: ○ —○, 2.38 mM; △ —△, 0.24 mM; ● —●, 0.05 mM. The inset shows replots of slopes and intercepts as functions of the reciprocal of the succinate concentration.



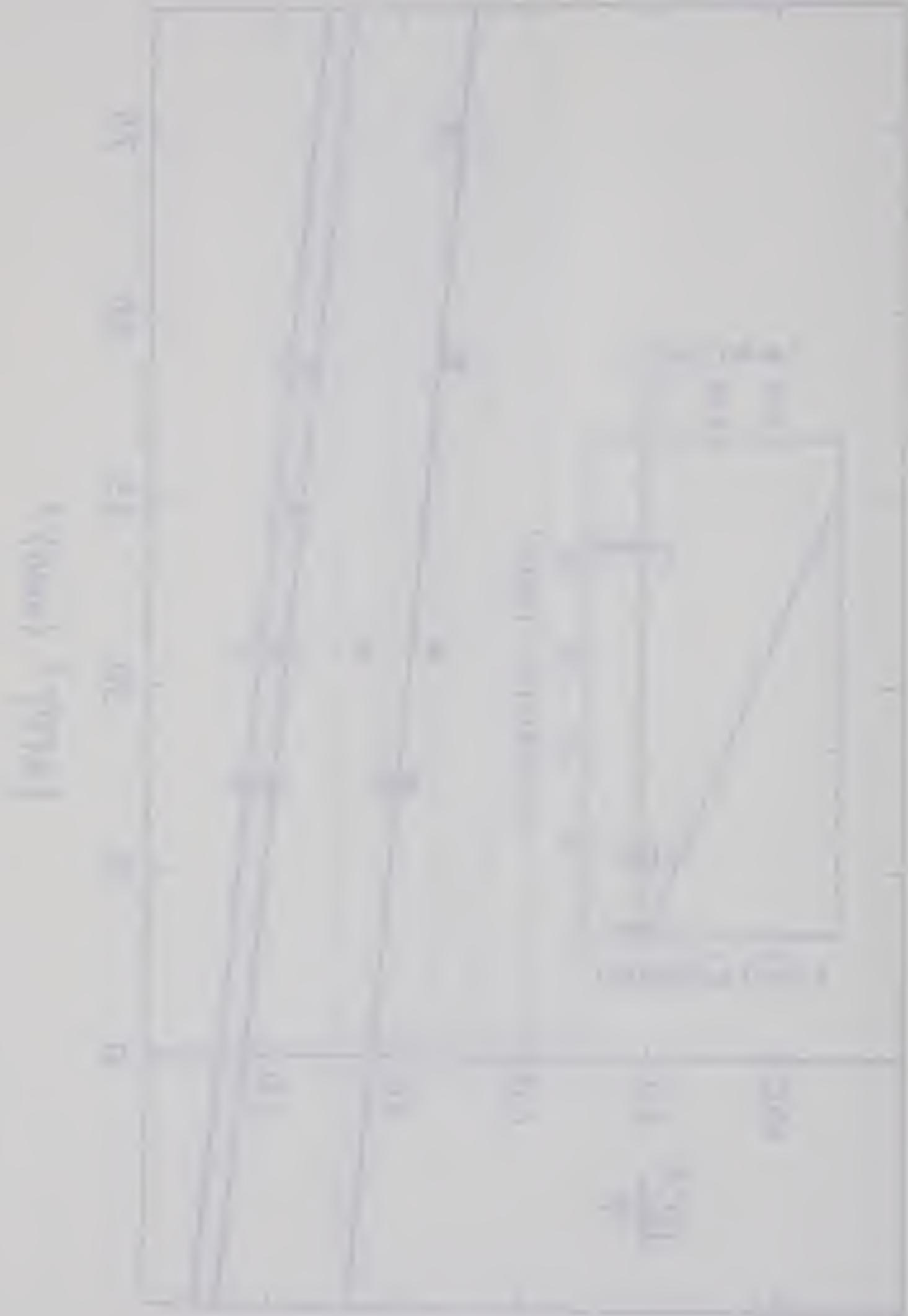




Figure 6. Reciprocal plots showing effects of varying ATP and CoA at a fixed non-saturating concentration of succinate. The reaction mixtures contained in a final volume of 3.09 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM  $MgCl_2$ , 1 mM dithiothreitol, 0.1 mM succinate, SCS (0.017 units) and CoA as follows:  $\circ$  —  $\circ$ , 10  $\mu M$ ;  $\Delta$  —  $\Delta$ , 4.0  $\mu M$ ;  $\bullet$  —  $\bullet$ , 2.1  $\mu M$ . The inset shows replots of slopes and intercepts as functions of the reciprocal of the CoA concentration.

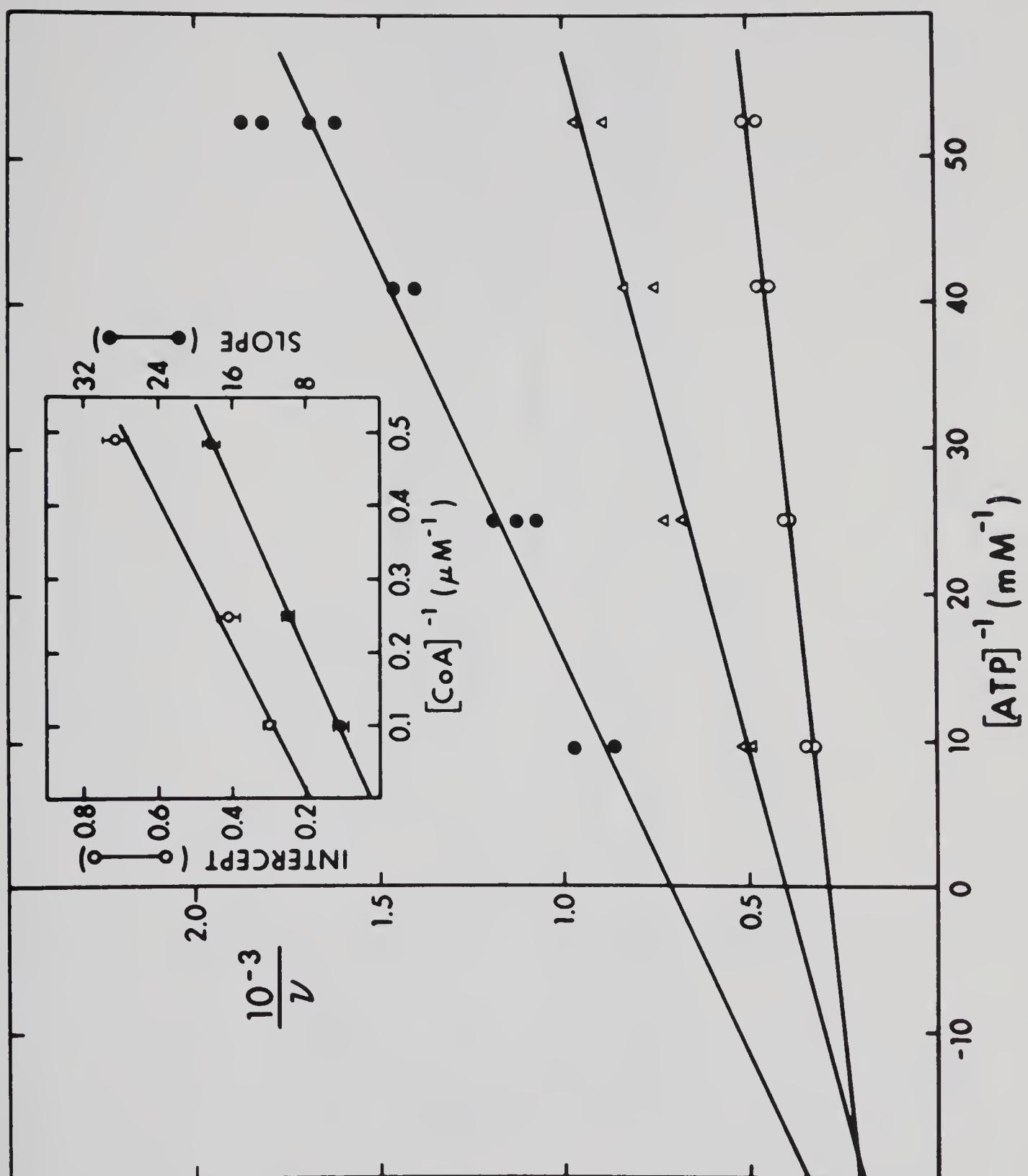






Figure 7. Reciprocal plots showing effects of varying ATP and CoA at a fixed near-saturating concentration of succinate. The reaction mixtures contained, in a final volume of 3.09 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.5 mM succinate, SCS (0.017 units) and CoA as follows: o —○—, 7.3 μM; • —●—, 4.6 μM; Δ —Δ—, 1.8 μM. The inset shows replots of slopes and intercepts as functions of the reciprocal of the CoA concentration.

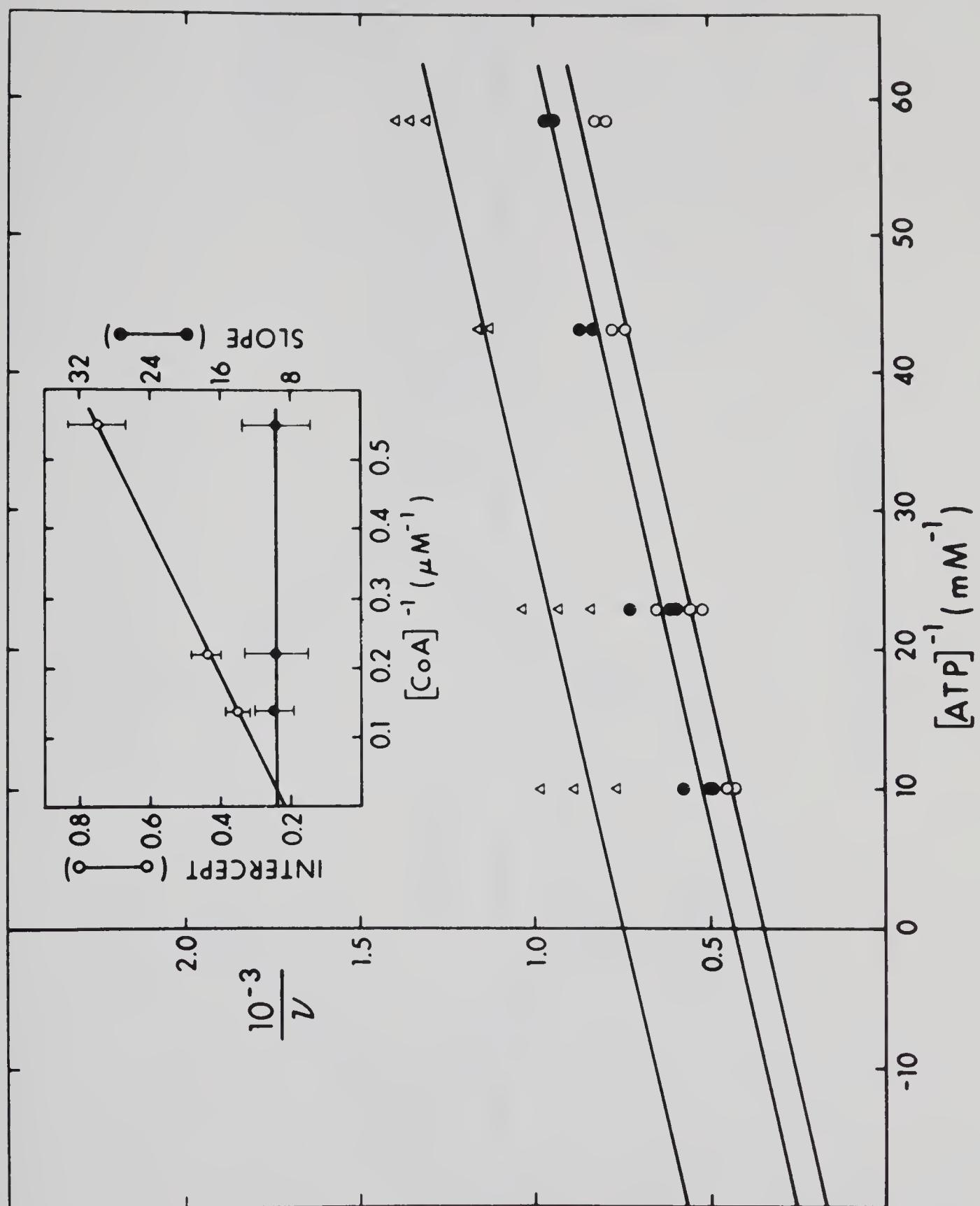
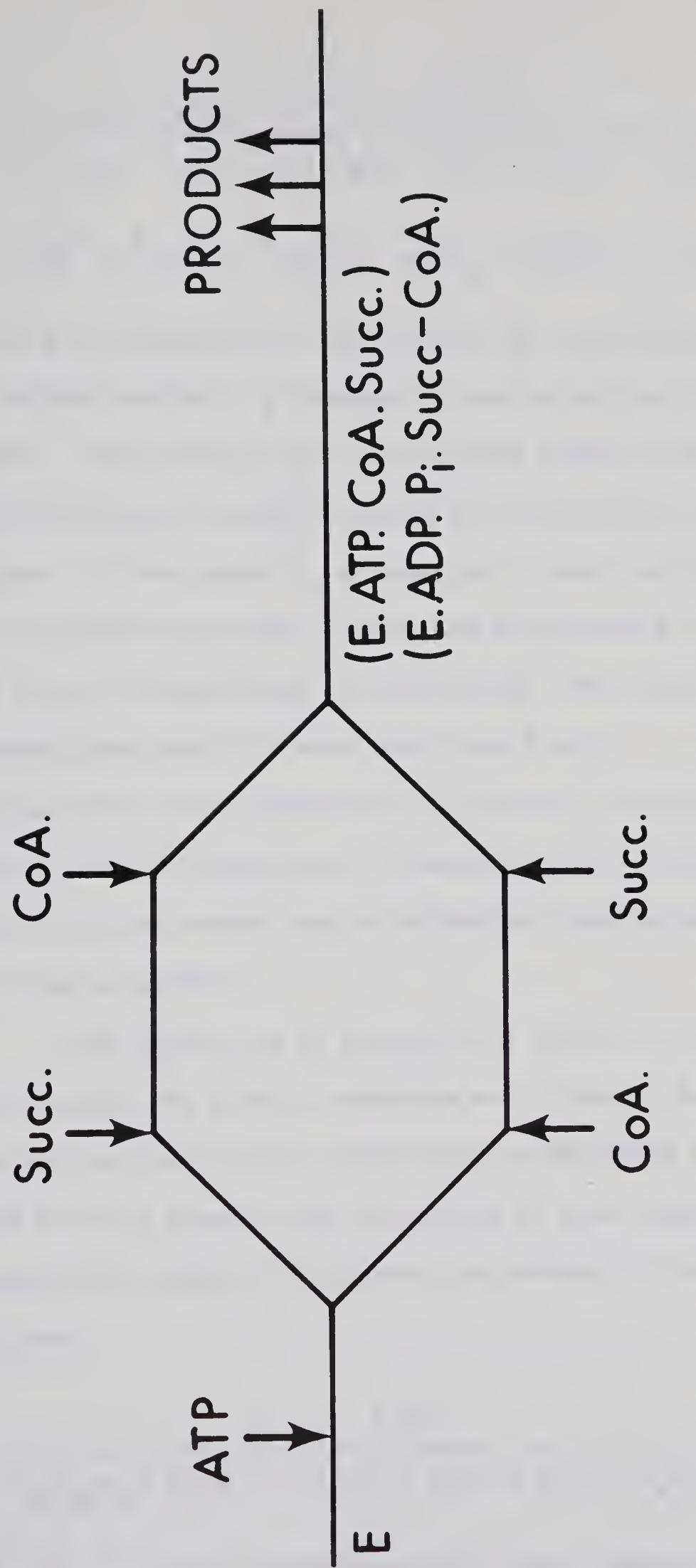






Figure 8. Mechanism I. Proposed reaction sequence for SCS in the direction of succinyl CoA formation.



MECHANISM I



$$v = \frac{E_\tau (\gamma_1^{ABC} + \gamma_2^{ABC^2} + \gamma_3^{AB^2C})}{\gamma_4 + \gamma_5^A + \gamma_6^B + \gamma_7^C + \gamma_8^{AB} + \gamma_9^{BC} + \gamma_{10}^{AC} + \gamma_{11}^{ABC}} [1]$$

$$+ \gamma_{12}^{AB^2} + \gamma_{13}^{AC^2} + \gamma_{14}^{B^2C} + \gamma_{15}^{BC^2} + \gamma_{16}^{AB^2C} + \gamma_{17}^{ABC^2}$$

A is the first substrate to add followed by the random addition of B and C in this system.  $\gamma_1$  through  $\gamma_{17}$  are collections of rate constants. This equation is in the second degree (47) in B and C and is not well suited to simplification to interpretable reciprocal equations. It does predict, as expected, linear reciprocal plots when A is the variable substrate, and curved plots when B or C is varied unless C or B, respectively, is saturating. This would imply that secondary plots should be non-linear when B or C, is the fixed variable substrate and C or B, respectively, is fixed, not saturating. Our failure to observe significant curvature in our secondary plots suggests that the random loop of MECHANISM I approximates a rapid equilibrium situation.

When either CoA or succinate is present at saturating concentrations, the kinetic mechanism would tend to follow either the top or bottom branch of the random loop of MECHANISM I. Therefore the ordered ter rate equation can be applied to this system. This equation in the absence of products, as derived by Cleland (38), is as follows:

$$v = \frac{V_1^{ABC}}{K_{ia} K_{ib} K_c + K_{ib} K_c A + K_{ia} K_b C + K_c^{AB} + K_b^{AC} + K_a^{BC} + ABC} [2]$$

where A, B, and C are, respectively the first, second, and third



substrates to add to the enzyme. When this equation is converted to reciprocal form with A as the variable substrate one obtains:

$$\frac{1}{V} = \frac{1}{V_1} \left( 1 + \frac{K_b}{B} + \frac{K_c}{C} + \frac{K_{ib} K_c}{BC} \right) + \frac{K_a}{AV_1} \left( 1 + \frac{K_{ia} K_b}{K_a B} + \frac{K_{ia} K_{ib} K_c}{K_a BC} \right) \quad [3]$$

The slope of this equation is then given by:

$$\text{Slope} = \frac{K_a}{V_1} \left( 1 + \frac{K_{ia} K_b}{K_a B} + \frac{K_{ia} K_{ib} K_c}{K_a BC} \right) \quad [4]$$

As has been pointed out (38,39) this slope will become equal to  $K_a/V_1$  when  $B \gg K_b$  and  $K_{ib}$ . This allows the calculation of  $K_{ATP} = 0.02 \text{ mM}$  from the data of figure 5 where  $B = \text{CoA} = 87 \mu\text{M}$ .

Rearrangement of equation [3] also shows that  $K_b$  may be calculated from tertiary plots of the y intercept of the secondary intercept replots against the reciprocal of the concentration of B, the second substrate to add. This comes from the relationship:

$$\text{INT} = \frac{1}{V_1} \left( 1 + \frac{K_b}{B} + \frac{K_c}{C} + \frac{K_{ib} K_c}{BC} \right) \quad [5]$$

This equation defines the secondary intercept replot and can be written:

$$\text{Intercept} = \frac{1}{V_1} \left( 1 + \frac{K_b}{B} \right) + \frac{K_c}{V_1 C} \left( 1 + \frac{K_{ib}}{BC} \right) \quad [6]$$

From this it follows that the intercept of this intercept plot will be:

$$\text{Intercept}_{\text{INT.}} = \frac{1}{V_1} + \frac{1}{B} \left( \frac{K_b}{V_1} \right) \quad [7]$$



The  $K_{CoA}$  was calculated from the tertiary intercept replot shown in Figure 9, using the data from figures 3, 4, and 5. This analysis gave a value of 1.5  $\mu\text{M}$  for  $K_{CoA}$ . The data of figures 6 and 7 and other similar experiments were analyzed in an identical manner to give a value for  $K_{Succ.}$  of 0.10 mM.

It should be pointed out that this analysis involves an approximation. The analysis is based on the ordered ter rate equation (Equation [2]) with the assumption that this system will fit such a model at saturating concentration of B or C. Obviously,  $3^\circ$  intercept replots will not arise if the fixed substrate is completely saturating in all the initial rate experiments from which they are derived. Nevertheless, the linearity of the  $3^\circ$  intercept replot of figure 17 suggests that the ordered ter equation is a good approximation to this system under all conditions used and the derived constants can be regarded with some confidence. This is another indication that the random loop of MECHANISM I approaches a rapid equilibrium condition.

The kinetic data reported above are clearly not those expected for the mechanisms of SCHEME I-IV (Chapter I) in which ADP is predicted to dissociate from the enzyme before subsequent binding of succinate and CoA. Further evidence against the formation of the E-P intermediate as a discrete step in catalysis has been obtained from a product inhibition study with ADP. If E-P is produced in a discrete step including ADP dissociation before subsequent binding of other substrates, then ADP would combine with E-P, and ATP with free enzyme in the overall reaction. Cleland's theory (38) predicts that in this case noncompetative product inhibition should be observed when ADP is





Figure 9. Replot, according to equation [7], of the  $\gamma$  intercepts of the intercept replots of figures 3, 4, and 5 versus the reciprocal of the CoA concentration.

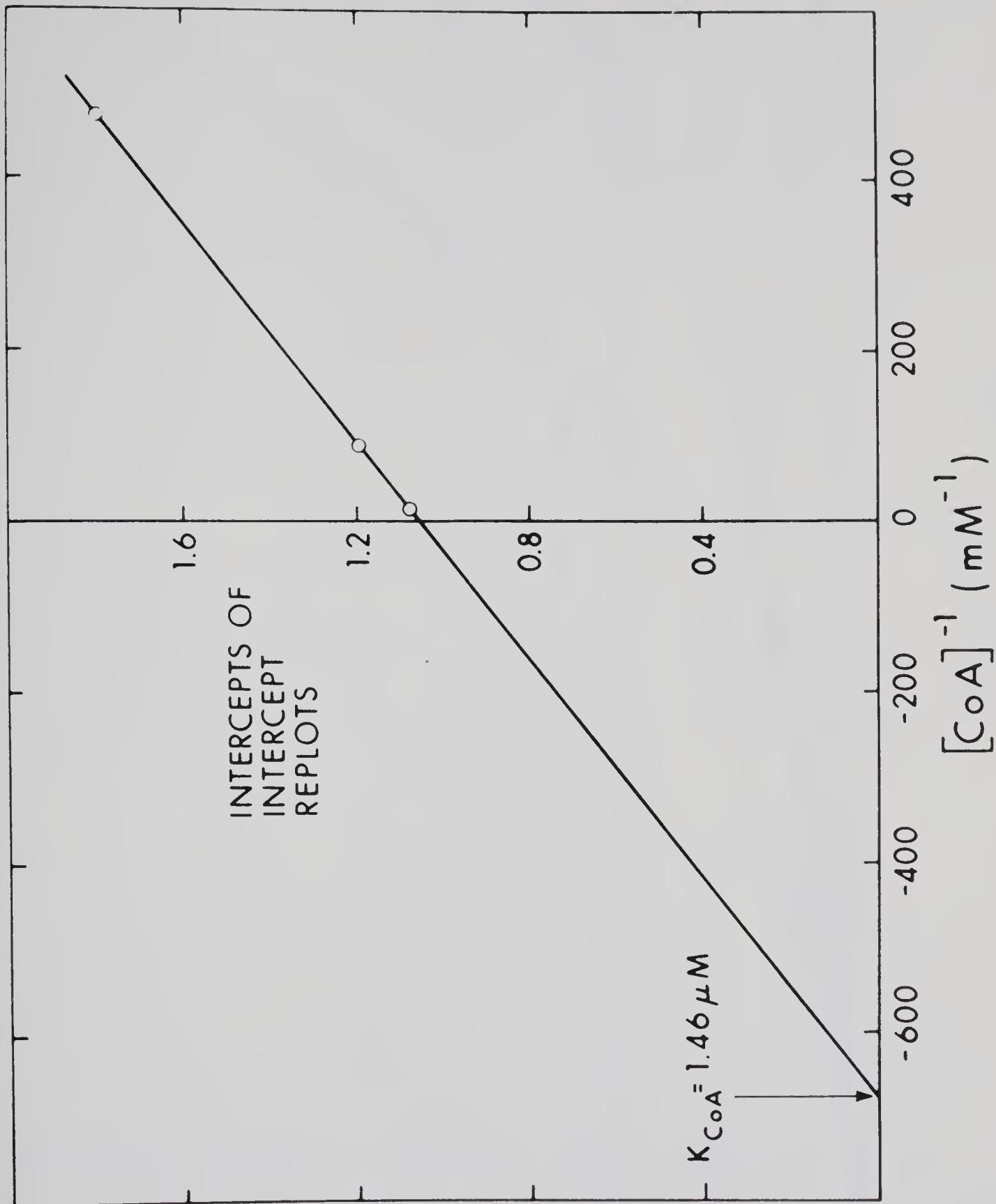






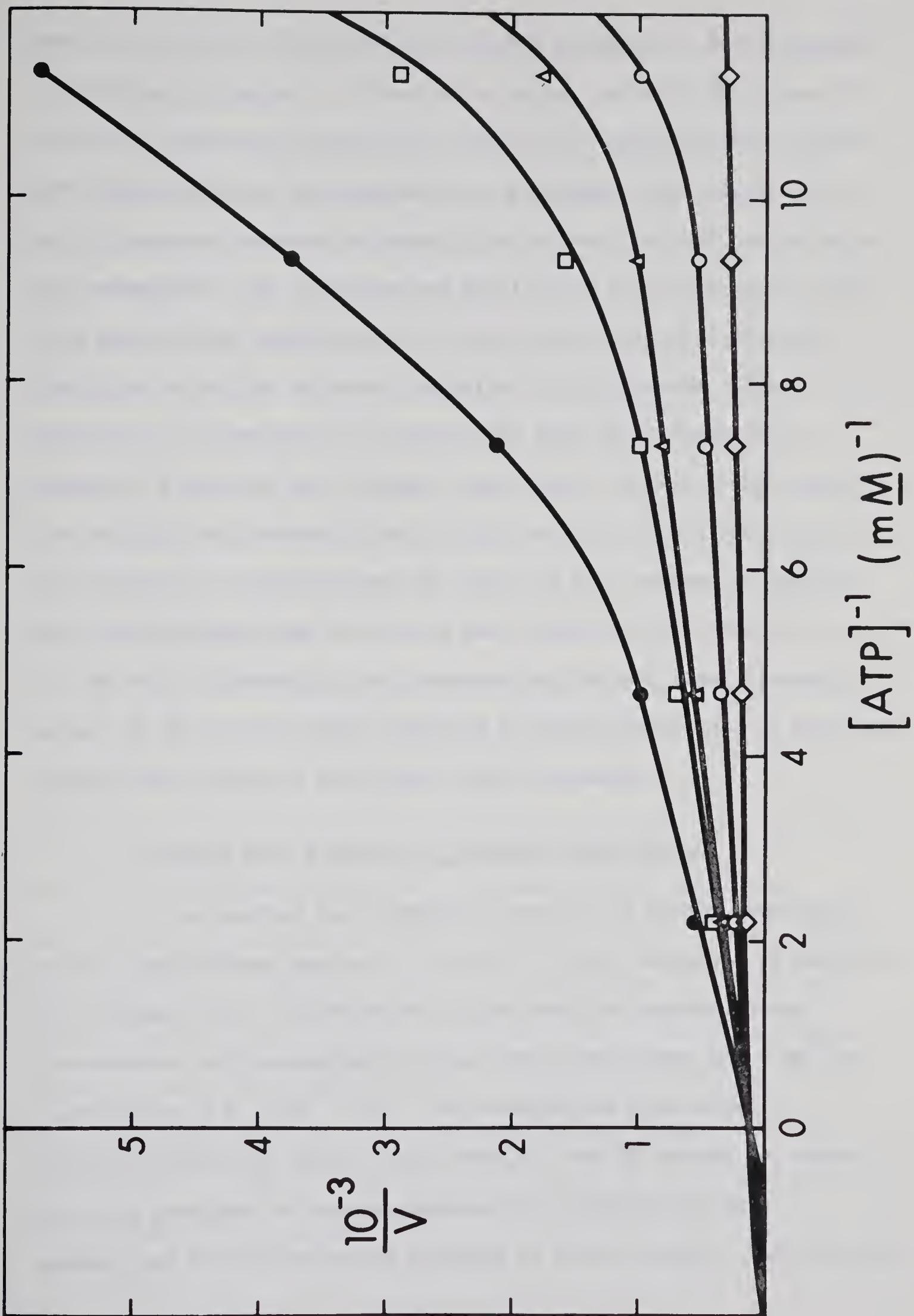
Figure 10.

Reciprocal plots showing product inhibition by ADP with ATP as the variable substrate.

The reaction mixtures contained in a final volume of 1.09 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM succinate, 10 mM MgCl<sub>2</sub>, 86 μM CoA, 0.038 units of SCS, and ADP as follows:

◇—◇, none; ○—○, 4 μM; Δ—Δ, 10 μM; □—□, 40 μM; ●—●, 100 μM.

Optical path length = 1 cm.





used as an inhibitor with ATP the variable substrate. This experiment is depicted in figure 10. There would appear to be little change of intercept, indicating competitive inhibition, suggesting that ADP and ATP combine with the same enzyme form, presumably free enzyme. The very pronounced curvature of these plots at very low ADP concentration was unexpected. The curvature may result from the combination of ADP at a second site, perhaps the CoA site, thus acting as a dead end inhibitor as well as a product inhibitor in this system. This possibility is supported by observations made while studying the kinetics of succinyl CoA cleavage (see below). It was found that at low succinyl CoA concentrations (5  $\mu$ M), substrate inhibition by ADP was observed at concentrations of about 0.3 mM. However at succinyl CoA concentrations near 150  $\mu$ M, no such inhibition was observed at 0.5 mM ADP. Alternately, the curvature may reflect some allosteric effect of ADP in the system, although no other indications of any such interactions have ever been noted with this enzyme.

## 2. Initial Rate Kinetics of Succinyl CoA Cleavage

The succinyl CoA synthetase reaction is freely reversible with an equilibrium constant of about 3.7 in the direction of succinyl CoA cleavage (3). The enzyme will also form the phosphorylated intermediate with approximately equal ease from either  $ATP + Mg^{++}$  or  $succinyl CoA + P_i + Mg^{++}$  (12). The observation, from product inhibition with ADP (above), that both ATP and ADP appear to combine with the same form of enzyme suggests the possibility that a symmetrical kinetic mechanism operates in this reaction. With this in



mind we have applied the preceding kinetic analysis to the succinyl CoA cleavage reaction.

Figure 11 shows the double reciprocal plot obtained when ADP, as the variable substrate, is plotted against  $P_i$ , as the fixed variable substrate, at a fixed concentration of succinyl CoA near its  $K_m$  (see below). The lines are fitted to the data by the method of Wilkinson (48) and may be drawn to a common intersection point within the standard error of their coefficients. The inset to figure 11 shows that the slope increases as the concentration of  $P_i$  decreases indicating that no product dissociates between the addition of ADP and  $P_i$  in the reaction sequence. The inset also shows that both slope and intercept of the primary plots are linear functions of the reciprocal of the  $P_i$  concentration.

When the concentration of succinyl CoA was raised to about five times the  $K_m$  the primary plots became parallel as shown in figure 12. The inset to figure 12 again shows the secondary replots which indicate that the slope is constant in all cases, and that the intercept is a linear function of the fixed variable substrate concentration. The experiment was repeated at a still higher succinyl CoA concentration (approximately  $15 \times K_m$ ) and figure 13 demonstrates that the plots remained parallel. The inset to figure 13 shows that secondary replots were again linear. In these plots, similar to the results obtained for the kinetics of succinyl CoA formation in part 1, the slope of the intercept replot decreases as the concentration of the fixed substrate increases.



These data are very similar to those for the analogous plots in the direction of succinyl CoA formation and suggest that succinyl CoA adds to the enzyme between the points of addition of ADP and  $P_i$  in a sequential manner to produce a quaternary complex.

Consideration of the systems in which ADP and succinyl CoA are varied at fixed concentrations of  $P_i$  suggest that an overall mechanism of the type shown in figure 8 (MECHANISM I) will also describe the reaction in the direction of succinyl CoA cleavage. Figure 14 shows the pattern for ADP and succinyl CoA varied at low  $P_i$  concentration (about  $1/4 K_m$ ). The plots are clearly intersecting and again will meet at common point within the standard errors of the fitted lines. The inset to figure 14 shows secondary replots as linear functions of the reciprocal of the succinyl CoA concentrations and emphasizes the change in slope with the change in succinyl CoA concentration. When  $P_i$  concentration is raised to a level of about  $3 \times K_m$ , the plot shown in figure 15 is obtained. The lines become parallel within experimental error as is confirmed by the slope replot shown in the inset to figure 15. The inset also indicates that intercepts are again linear functions of the fixed variable substrate concentration. In this case as well the intercepts are less strong a function of the succinyl CoA concentration at the higher  $P_i$  concentration.

Thus it would appear that in this case succinyl CoA and  $P_i$  are kinetically equivalent, analogous to CoA and succinate in the succinyl CoA formation reaction. Therefore either can be the second substrate to add in a sequential addition sequence and MECHANISM I will also describe the succinyl CoA cleavage reaction. Figure 16 shows this mechanism written for the cleavage reaction.





Figure 11. Reciprocal plots showing effects of varying ADP and  $P_i$  at a fixed non-saturating concentration of succinyl CoA. The reaction mixtures contained in a final volume of 3.20 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM MgCl<sub>2</sub>, 5.3  $\mu$ M succinyl CoA, SCS (0.035 units) and  $P_i$  as follows:  $\blacktriangle$  —  $\blacktriangle$ , 6.39 mM;  $\circ$  —  $\circ$ , 1.92 mM;  $\Delta$  —  $\Delta$ , 0.64 mM. The Inset shows replots of the intercepts and slopes as functions of the reciprocal of the  $P_i$  concentration.

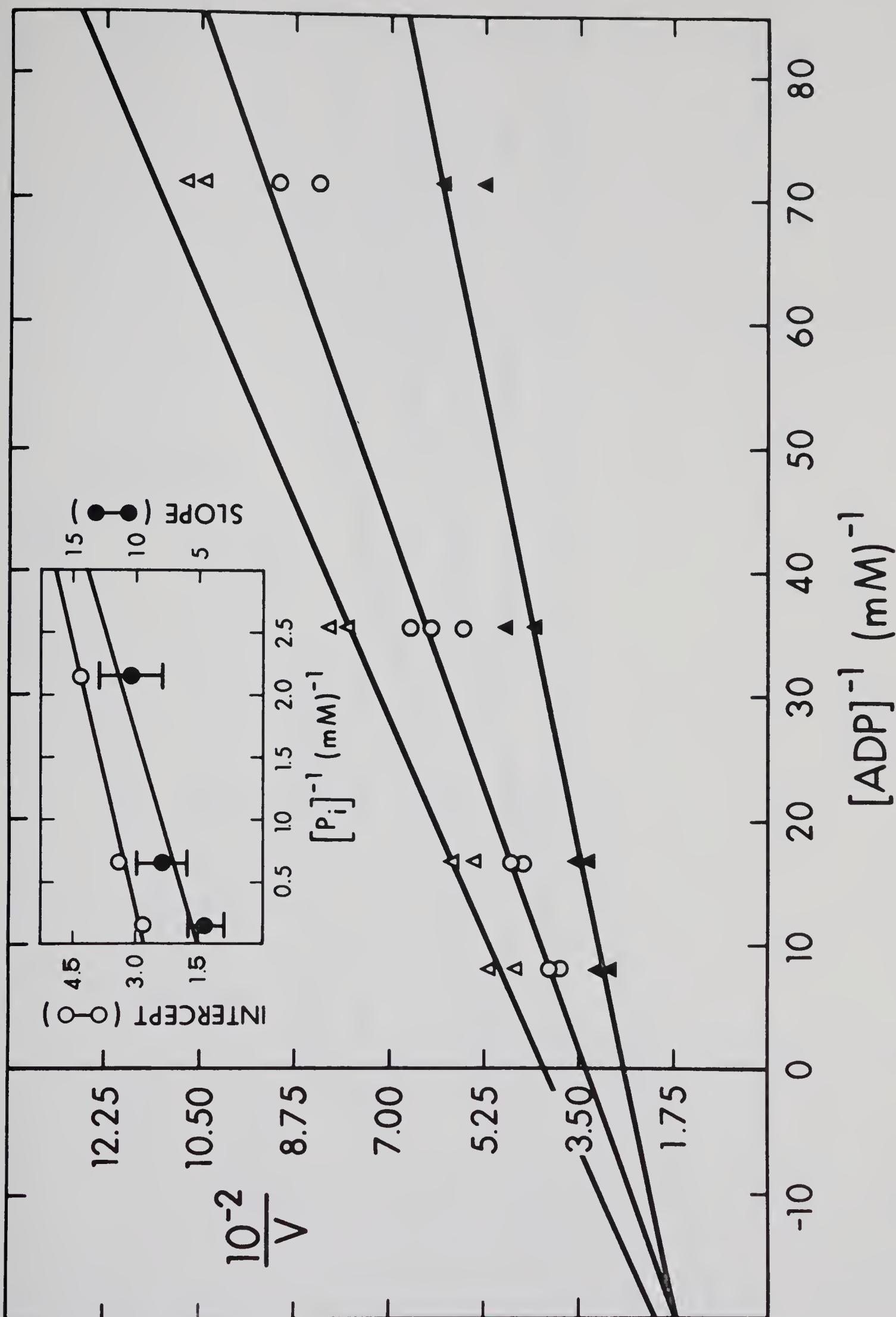






Figure 12. Reciprocal plots showing effects of varying ADP and  $P_i$  at a fixed near-saturating concentration of succinyl CoA. The reaction mixtures contained in a final volume of 3.20 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM  $MgCl_2$ , 46  $\mu M$  succinyl CoA, SCS (0.035 units), and  $P_i$  as follows:  $\blacktriangle$  —  $\blacktriangle$ , 6.14 mM;  $\circ$  —  $\circ$ , 1.54 mM;  $\triangle$  —  $\triangle$ , 0.46 mM. The inset shows replots of slopes and intercepts as functions of the reciprocal of the  $P_i$  concentration.

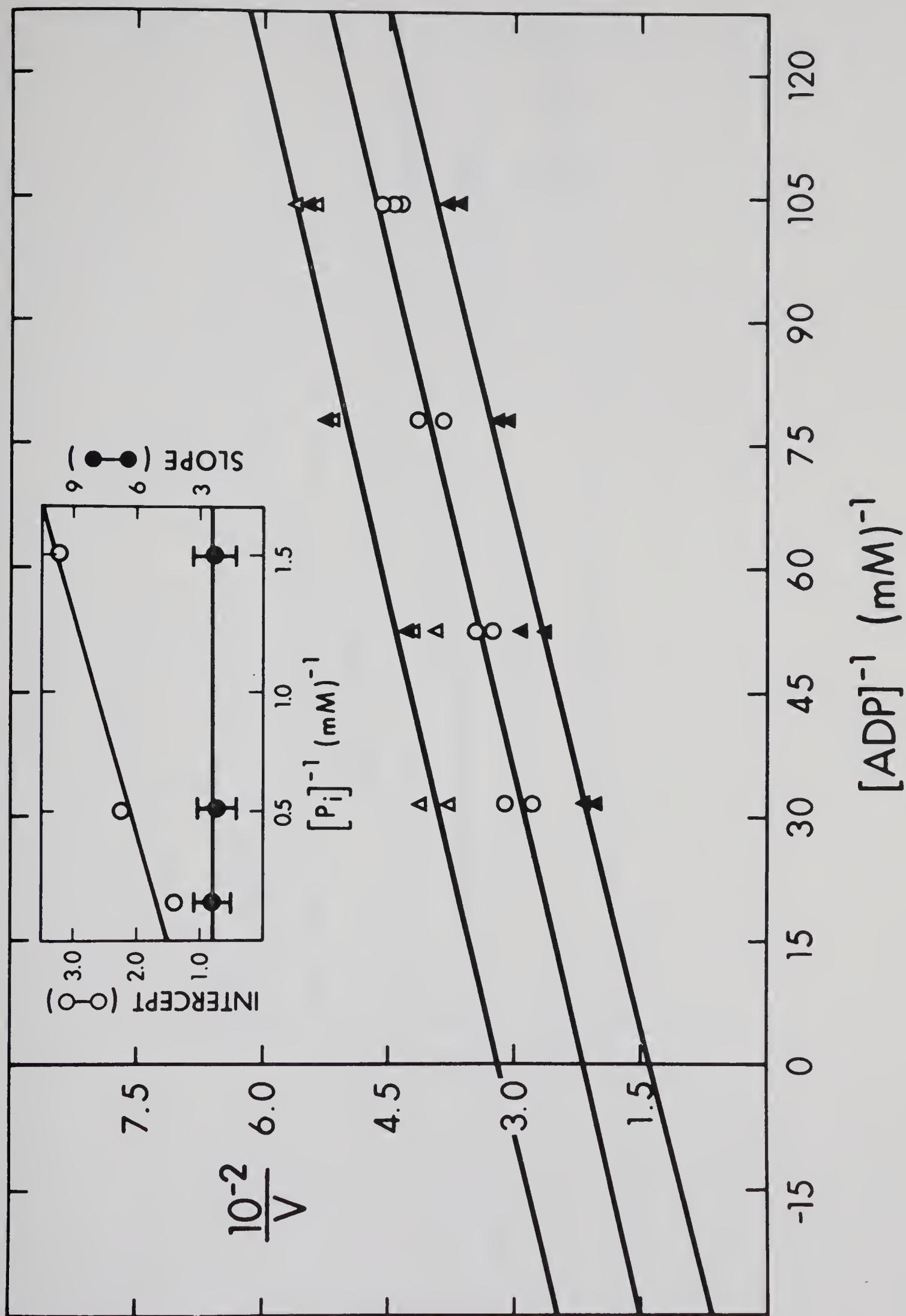






Figure 13. Reciprocal plots showing effects of varying ADP and  $P_i$  at a fixed, saturating concentration of succinyl CoA. The reaction mixtures contained in a final volume of 1.10 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM  $MgCl_2$ , 148  $\mu M$  succinyl CoA, SCS (0.012 units) and  $P_i$  as follows:  $\blacktriangle$  —  $\blacktriangle$ , 1.4 mM;  $\circ$  —  $\circ$ , 0.46 mM;  $\triangle$  —  $\triangle$ , 0.23 mM. Optical pathlength = 1 cm. The inset shows replots of slopes and intercepts as functions of the reciprocal of the  $P_i$  concentration.

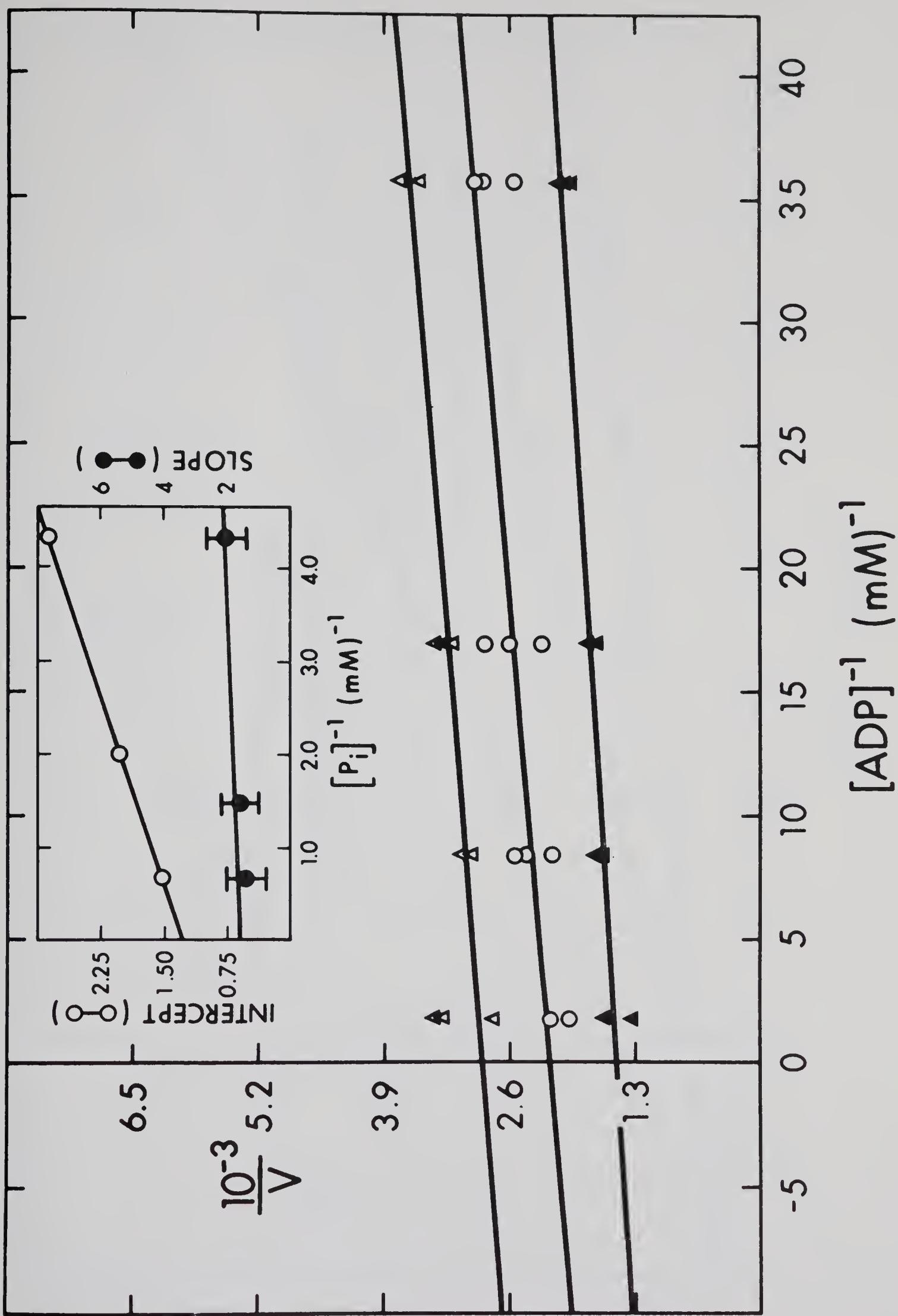






Figure 14. Reciprocal plots showing effects of varying ADP and succinyl CoA at a fixed non-saturating concentration of  $P_i$ . The reaction mixtures contained in a final volume of 3.15 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM  $MgCl_2$ , 0.65 mM  $P_i$ , SCS (0.035 units), and succinyl CoA as follows:  $\blacktriangle$  —  $\blacktriangle$ , 13.7  $\mu\text{M}$ ;  $\circ$  —  $\circ$ , 6.9  $\mu\text{M}$ ;  $\triangle$  —  $\triangle$ , 2.7  $\mu\text{M}$ . The inset shows replots of slopes and intercepts as functions of the reciprocal of the succinyl CoA concentration.

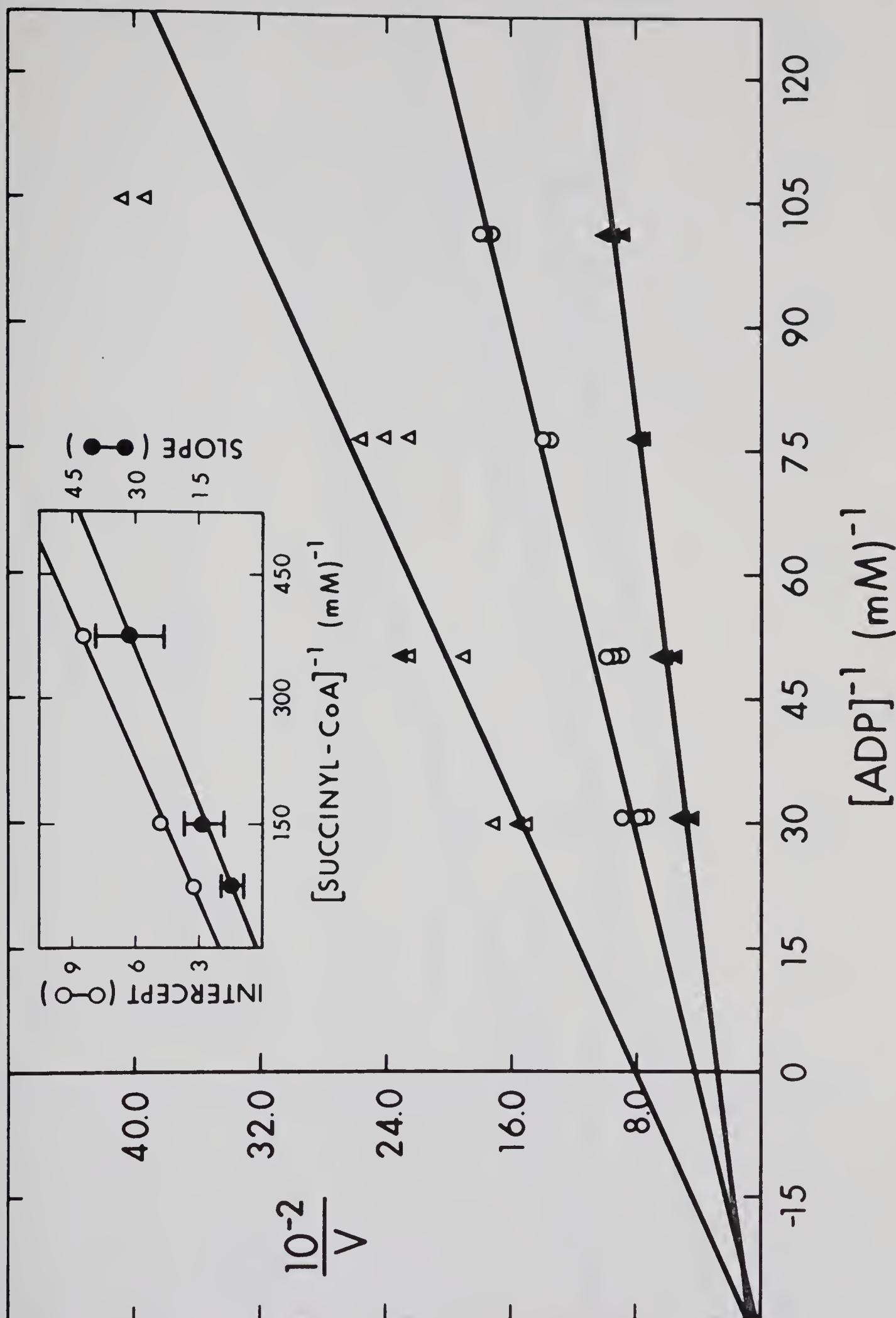






Figure 15. Reciprocal plots showing effects of varying ADP and succinyl CoA at a fixed near-saturating concentration of  $P_i$ . The reaction mixtures contained in a final volume of 3.15 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM  $MgCl_2$ , 6.65 mM  $P_i$ , SCS (0.035 units) and succinyl CoA as follows:  $\blacktriangle$  —  $\blacktriangle$ , 13.3  $\mu M$ ;  $\circ$  —  $\circ$ , 6.7  $\mu M$ ;  $\triangle$  —  $\triangle$ , 2.7  $\mu M$ . The inset shows replots of slopes and intercepts as functions of the reciprocal of the succinyl CoA concentration.

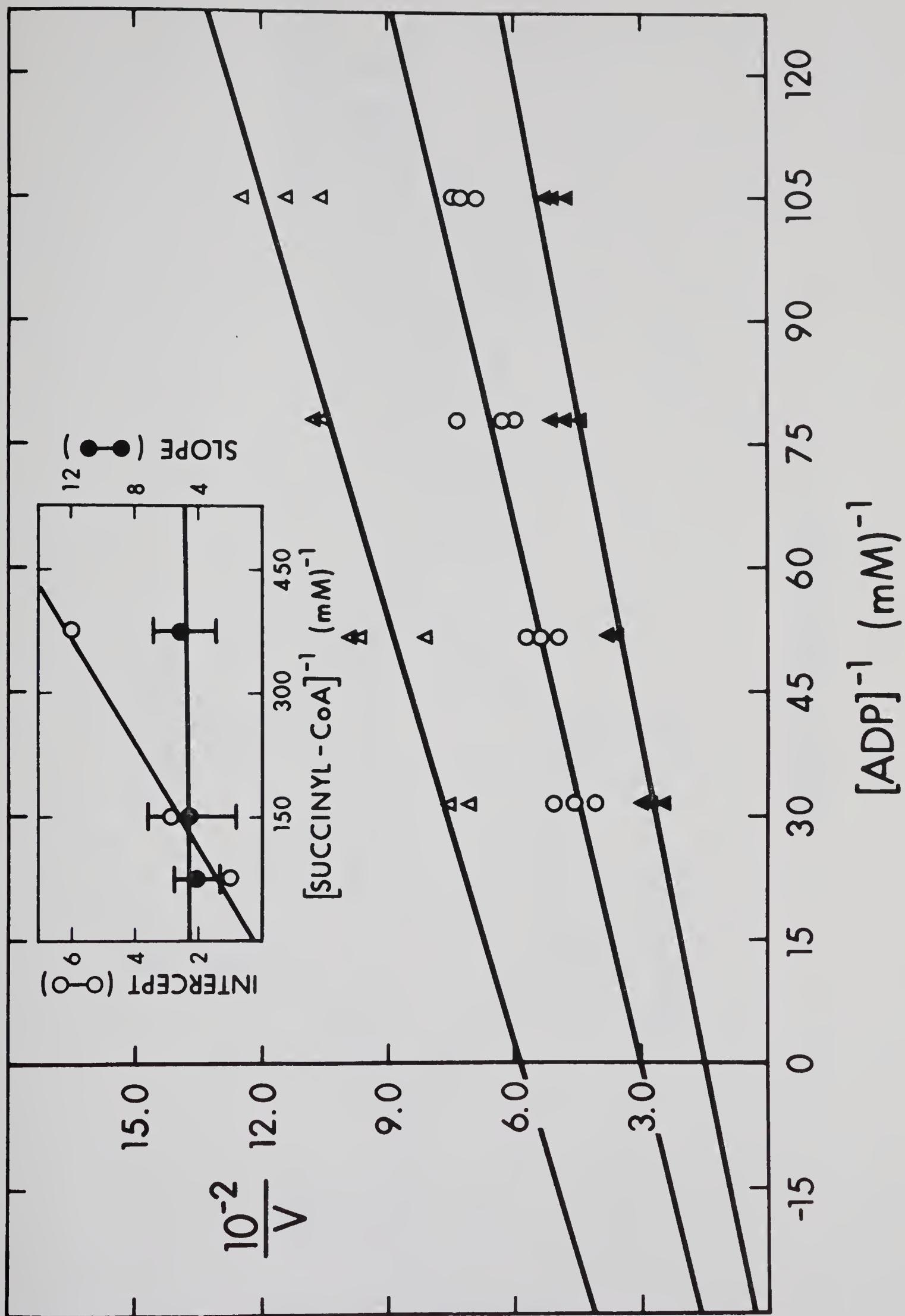
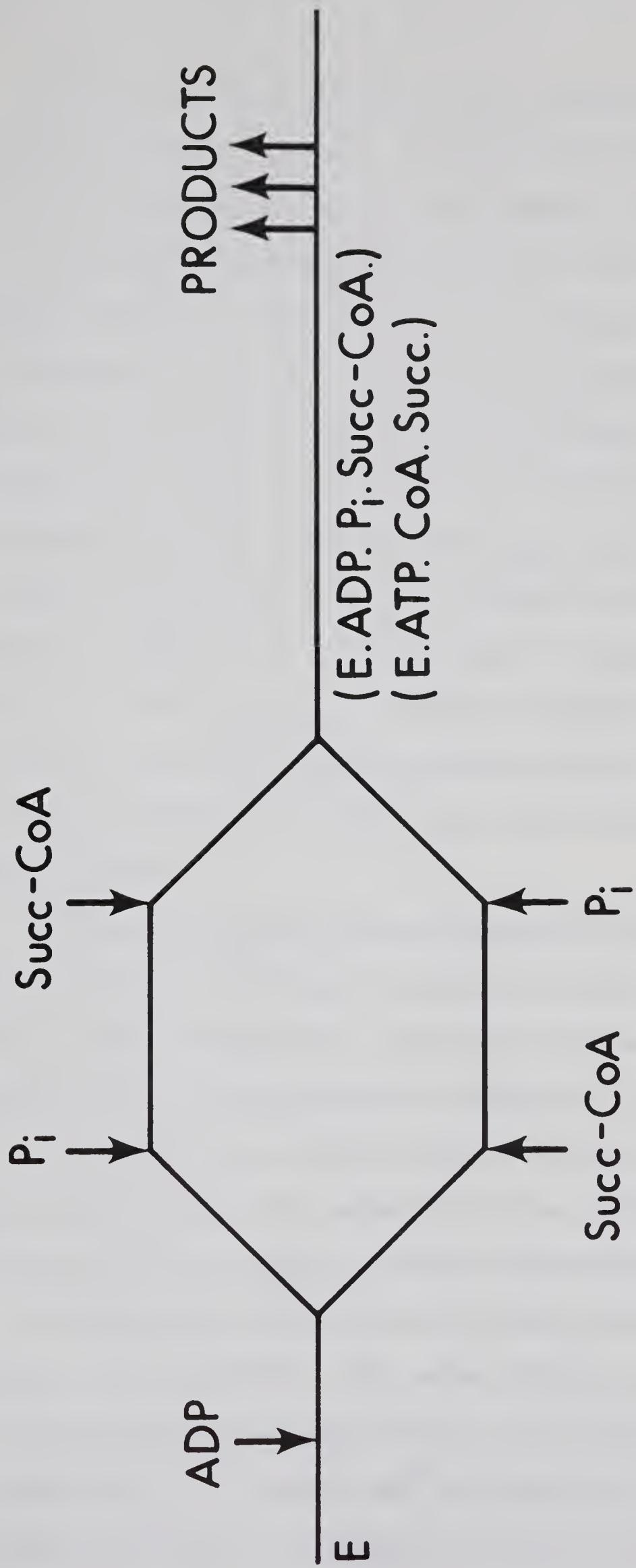






Figure 16. Mechanism II. Proposed reaction sequence for SCS in the direction of succinyl CoA cleavage.



## MECHANISM II



Equation [1] will thus hold for the initial velocity in either direction of this reaction. It follows that the same arguments concerning the use of the ordered ter rate equation, discussed in part 1, to approximate this system should hold for the reaction in the cleavage direction. Since the slope of a primary plot of  $1/V$  vs  $1/A$  at saturating B will go to a value of  $K_a/V_1$  according to this analysis, we can calculate a  $K_{ADP}$  of  $12 \mu M$  from figure 13 where  $B = \text{succinyl CoA} = 148 \mu M$ . We can also use the tertiary intercept replot analysis described in part 1 to get  $K_{\text{succ-CoA}}$  and  $K_{P_i}$ . Such a replot of intercepts of intercept replots for the experiments of figures 11, 12, and 13 is shown in figure 17. It gives a value of  $7.7 \mu M$  for  $K_{\text{succ-CoA}}$ . A similar treatment of the data of figures 14 and 15 and other similar experiments gives a  $K_{P_i}$  value of  $2.6 \text{ mM}$ . The  $K_m$  values for all substrates in both directions are summarized in Table I.

The overall apparent kinetic mechanism of succinyl CoA synthetase then can be described by a partially random sequential model, MECHANISM III shown in Figure 18. The kinetic results definitely are not compatible with a ping-pong kinetic mechanism. As pointed out in Chapter I, this result is in harmony with the substrate synergism phenomena associated with this enzyme, but is not ordinarily to be expected for a system involving a covalent enzyme-substrate intermediate.

The enzyme ATP-citrate lyase bears some mechanistic resemblance to SCS (57,58,59). This enzyme catalyzes the cleavage of citrate to oxaloacetate and acetyl-CoA with the concomitant degradation of ATP to ADP and  $P_i$ . It requires  $Mg^{++}$  and shares other common features with the succinyl CoA synthetase reaction, including the





Figure 17. Replot, according to equation [7], of the  $\gamma$  intercepts of the intercept replots of figures 11, 12, and 13 versus the reciprocal of the succinyl CoA concentration.

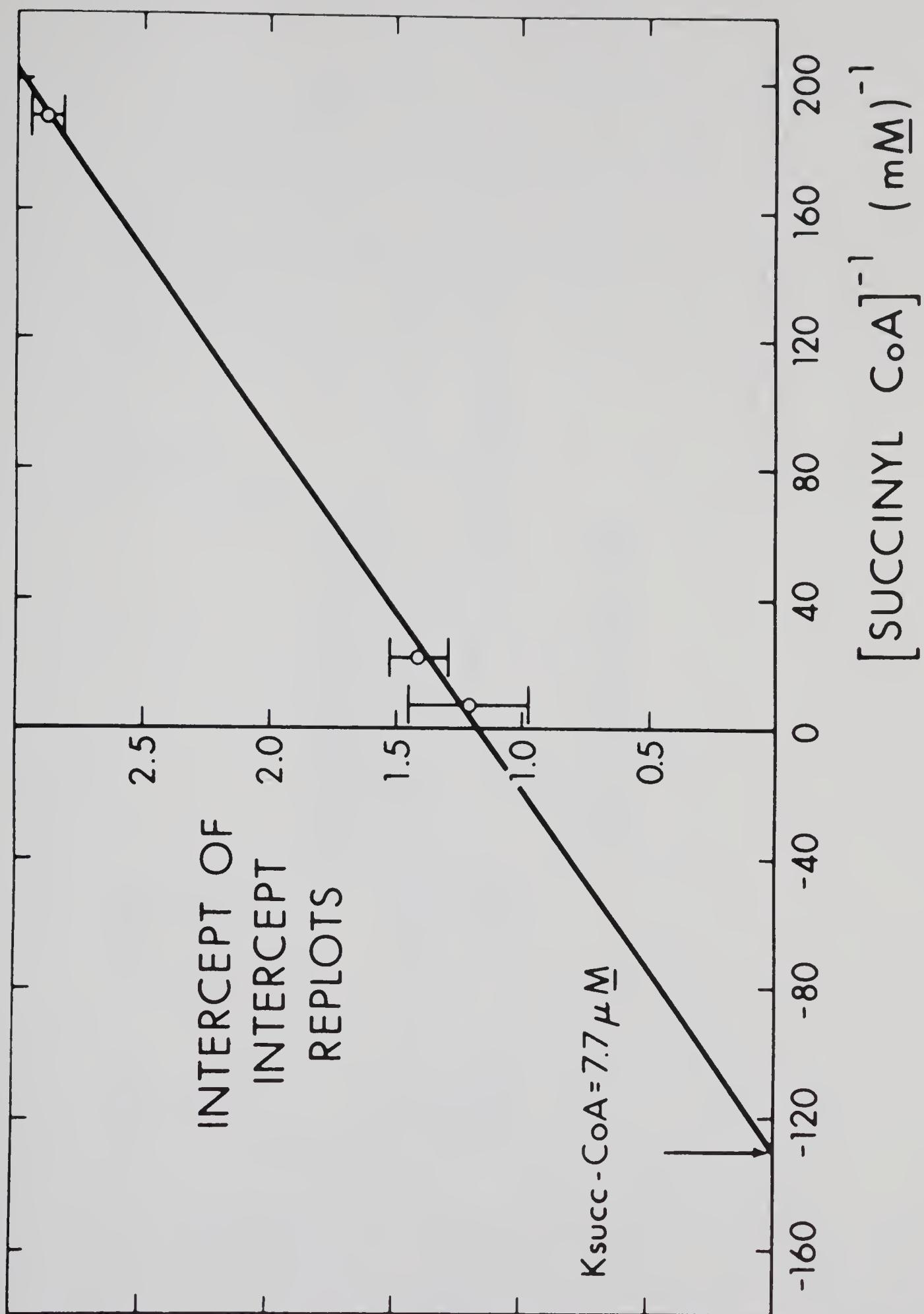
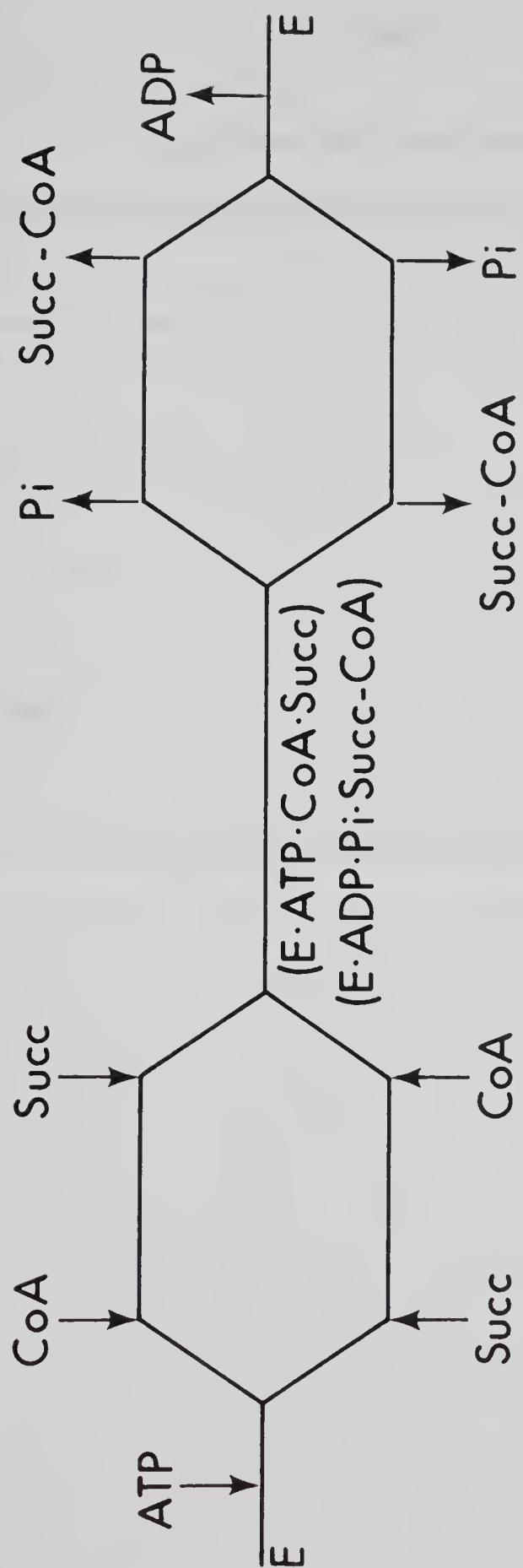






Figure 18. Mechanism III. Proposed partially random sequential mechanism for the overall reversible SCS reaction.



### MECHANISM III



TABLE I

 $K_m$  Values for Substrates of SCS

Substrate	$K_m$ (M)
ATP	$2 \times 10^{-5}$
Succinate	$1 \times 10^{-4}$
CoA	$1.5 \times 10^{-6}$
ADP	$1.2 \times 10^{-5}$
Succinyl CoA	$7.7 \times 10^{-6}$
$P_i$	$2.6 \times 10^{-3}$



formation of a phosphorylated enzyme (57,58) which may occur via phosphohistidine (16,59). Plowman and Cleland (57) have investigated the initial rate kinetics of the enzyme and have proposed a mechanism similar to MECHANISM I (figure 8) with ATP combining first followed by random addition of citrate and CoA. They also found that this enzyme catalyzes an  $\text{ATP} \longleftrightarrow \text{ADP}$  isotope exchange reaction in the absence of other substrates at about the same rate as in the presence of other substrates. These authors interpret this result to mean that a minor ping-pong pathway exists in the kinetic mechanism. In the case of succinyl CoA synthetase the substrate synergism effects in  $\text{ATP} \longleftrightarrow \text{ADP}$  exchange in conjunction with the initial rate kinetic patterns argue against a significant ping-pong contribution to the kinetic mechanism. In addition, the participation of the phospho-enzyme of the citrate cleavage reaction as a catalytic intermediate has not been established. In fact, Plowman and Cleland (57) found that enzyme-phosphates formed from opposite directions of the reaction appeared to have different properties.

Succinyl CoA synthetase from E. coli is, then, to our knowledge, the only case so far reported in which an enzyme known to involve a covalent intermediate in catalysis has been shown to exhibit other than ping-pong kinetics. The case of SCS of pig-heart should however be mentioned. Cha et al. reported a study of the kinetics of this enzyme on a partially purified preparation in 1964 (60). They proposed a mechanism involving random GTP and CoA addition to the enzyme followed by addition of succinate. This work was done before the discovery that the enzyme could form a phosphorylated intermediate. However, these authors have subsequently suggested (21) that the majority



of their kinetic data can be accommodated to this result. At any rate the kinetics are clearly not compatible with a simple ping-pong mechanism. Although it appears likely that the pig-heart SCS will resemble the *E. coli* enzyme in displaying some form of sequential mechanism it must be emphasized that in this case the participation of the enzyme-phosphate species in catalysis has not as yet been unequivocally demonstrated.

### C. Summary

Succinyl coenzyme A synthetase of *Escherichia coli* does not exhibit a steady state kinetic pattern indicative of ping-pong kinetics, despite the fact that the participation of a phosphorylated enzyme covalent intermediate has been established. Instead, kinetic patterns are consistent with the sequential addition of all substrates, to form a quaternary complex, before the release of any product.

Double reciprocal plots are intersecting with ATP and succinate varied at non-saturating concentration of the third substrate, CoA. These plots become parallel at higher CoA concentrations suggesting that CoA can be the second substrate to add in the binding sequence. Analogous reciprocal plots with ATP and CoA varied at both non-saturating and saturating succinate concentrations are also intersecting and parallel, respectively. The data may be reconciled with a binding sequence in which ATP binds to the enzyme first, followed by the random addition of CoA and succinate.



Similar initial rate kinetic studies in the direction of succinyl CoA cleavage give analogous patterns on double reciprocal plots suggesting that in this direction a sequential mechanism, involving initial ADP binding followed by random addition of succinyl CoA and inorganic phosphate, also operates. Product inhibition by ADP with ATP as the variable substrate suggests that these nucleotides combine with the same form of the enzyme in the reaction sequence. A partially random sequential mechanism for the overall reversible reaction which accounts for all the kinetic results is proposed.  $K_m$  values for all substrates in both directions are calculated from the kinetic data.

The finding of a sequential mechanism for the addition of substrates to this enzyme, whose catalysis is known to involve a covalent intermediate, serves to illustrate a possible danger in the use of initial rate kinetics. It is not possible to discount chemical mechanisms involving covalent intermediates by failure to observe the ping-pong kinetic patterns which may be anticipated for these catalytic routes.



## CHAPTER IV

## KINETIC MECHANISM OF SUCCINYL CoA SYNTHETASE



ISOTOPE EXCHANGE

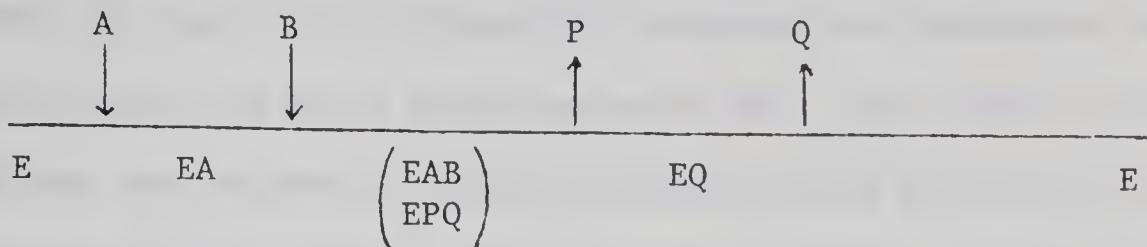
A. Introduction

The initial rate kinetic experiments described in the previous chapter define a partially random sequential kinetic mechanism for succinyl CoA synthetase and this must be a major pathway of the catalytic process. However, these studies are not of sufficient sensitivity to rule out the possible operation of minor pathways under various conditions. A more sensitive probe of reaction mechanism is the study of the kinetics at equilibrium in the absence of net chemical change made possible by the use of isotopically labelled substrates.

The use of isotope exchange at equilibrium in the study of enzyme mechanisms was first proposed by Boyer and his associates (36,37). They developed theoretical relationships for the bi-substrate case and illustrated the technique in the study of lactate dehydrogenases (61) and yeast hexokinase (62). In sequential mechanisms the method provides a means of distinguishing random from ordered binding sequences, gives information concerning the order of substrate addition and product release and can demonstrate whether or not the intraconversion of ternary complexes is rate limiting. Cleland has reviewed the application of isotope exchange in kinetic studies and has presented a method for the derivation of rate equations for more complicated mechanisms (63,64).



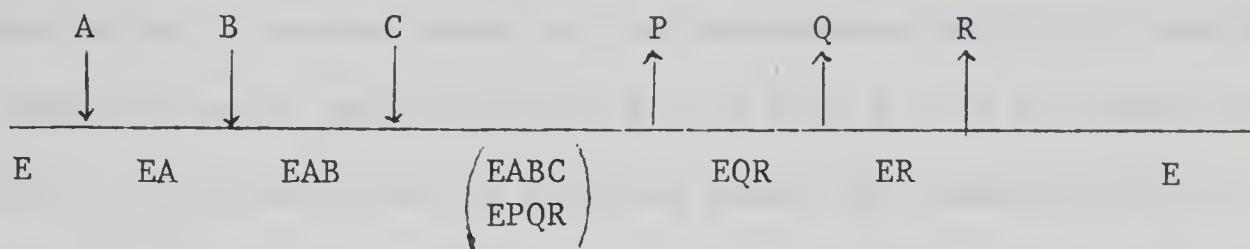
The widest application of this technique has been in the delineation of random or ordered mechanisms in bi-substrate reactions. The ordered mechanism can be written in Cleland's terminology as:



The rate equations for isotope exchange in such a system, as presented by Boyer and Silverstein (37), predict that the rate of A  $\longleftrightarrow$  Q exchange will go to zero as the concentration of B + P is raised toward infinity in constant ratio. This is a consequence of the fact that high concentrations of B and P can prevent dissociation of A and Q from the central complex in an ordered mechanism. In a random mechanism no substrate inhibition should be observed for any exchange. Cleland has expounded this principle in more general terms, stating that in ordered reaction sequences, "total substrate inhibition is observed whenever a reactant whose concentration is being varied adds between the points of combination in the reaction sequence of the molecules between which exchange is being measured" (63). The principle applies to ping-pong as well as sequential kinetic mechanisms. However here the exchange reactions can be studied in separate partial reactions in the absence of some substrates. Also, in a ping-pong mechanism the rate of exchange of a substrate-product pair in one partial reaction can be inhibited by raising the concentration of another substrate-product pair in the other partial reaction as a consequence of enzyme distribution.



Although the rate equations will be quite complex the above principles can be applied qualitatively to a ter-reactant mechanism as proposed for succinyl CoA synthetase. The mechanism proposed in Chapter III (figure 18) requires the compulsory initial binding of ATP and final dissociation of ADP. The order of binding of substrates and release of products between these points are irrelevant to present considerations of the kinetics of  $\text{ATP} \rightleftharpoons \text{ADP}$  exchange and the mechanism can be reduced to the ordered ter form. This can be written:



SCHEME A

where A and R are ATP and ADP respectively, B and C can be either succinate or CoA and P and Q similarly can be either succinyl CoA or  $\text{P}_i$ .

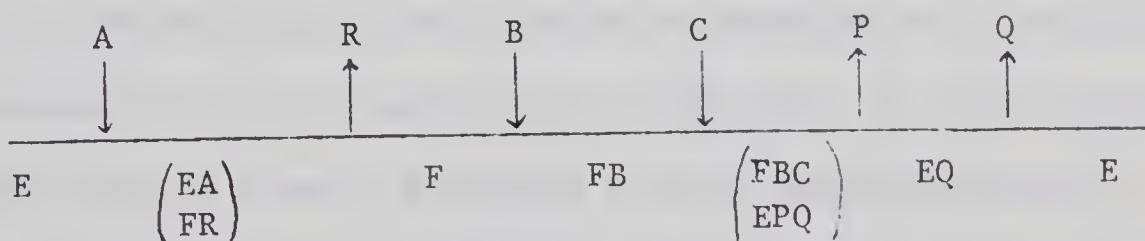
An equation can be derived by the method of Cleland (63) for  $V^*$ , the initial velocity of appearance of labelled R from labelled A. The derivation is based on the steady state assumption and is independent of whether or not the system is at chemical equilibrium. For the ordered ter system of scheme A this equation has the form:

$$\begin{aligned}
 V^* &= \frac{V_1 A^* BC (E/E_T)}{\frac{K_{ia} K_b K_c + K_{ia} K_b C + K_a BC + \frac{K_{ia} K_{ib} K_c K_q}{K_{iq} K_p}}{K_{iq} K_p} P} \quad [1] \\
 &+ \frac{K_{ia} K_{ib} K_c K_r}{K_p K_{iq} K_{ir}} PQ
 \end{aligned}$$



$A^*$  is the concentration of labelled A; B, C, P, and Q represent substrate and product concentrations as defined for SCHEME A, above.  $E/E_T$  is the free enzyme distribution relationship for the overall ter-ter kinetic mechanism as derived by Cleland (38), K and  $K_i$  values are kinetic constants for this mechanism also as defined by Cleland (38).  $V_1$  is the maximum initial velocity of product formation in the ordered ter reaction. The complete equation in terms of kinetic constants can be obtained by substituting the distribution expression,  $E/E_T$ , in equation [1]. This gives a very complicated expression with a multiplicity of squared terms in the denominator which will predict that  $V^*$  goes to zero as any pairing of B or C with P or Q is raised towards infinity. This would be the expected result by consideration of the fact that the binding of any of these pairs occurs between the points of combination of the exchanging reactants and would thus prevent exchange when they were raised to high concentration, as discussed above.

A ping-pong mechanism of the type that was ruled out on the basis of initial rate kinetic data would also result in complete inhibition of ATP  $\longleftrightarrow$  ADP exchange at high concentrations of other substrates. This mechanism has the form:



SCHEME B

where A and R are ATP and ADP respectively and the other letters refer to substrates and products as outlined for scheme A above. An



expression for the rate of  $A \longleftrightarrow R$  exchange,  $V^*$  can be derived by Cleland's method:

$$V^* = \frac{K_1 K_3 A^* (E)}{K_2 + K_3} \quad [2]$$

where  $A^*$  is the concentration of labelled A and  $K_1$ ,  $K_2$ ,  $K_3$  are rate constants for individual steps in the reaction. (Rate constants are numbered consecutively with the odd ones normally being for the forward reaction and the even ones for the reverse reaction, after the method of Cleland (64)). E is the concentration of free enzyme. This is clearly identical in form with the Michaelis-Menten equation and expresses the dependence of the rate on the distribution of E. Under initial velocity conditions when all enzyme is in the E form the rate  $V^*$  will be of course be equal to the initial velocity. At chemical equilibrium  $V^*$  will be a function of E distribution and as any pair of B or C with P or Q is raised to saturation, E will be complexed in the second half-reaction of scheme B;  $V^*$ , the rate of  $ATP \longleftrightarrow ADP$  exchange will be reduced to zero. With these considerations in mind we will consider the results of a study of the kinetics of  $ATP \longleftrightarrow ADP$  exchange in the succinyl CoA synthetase reaction.

#### B. Results and Discussion

Figure 19 shows the results of an experiment wherein  $ATP \longleftrightarrow ADP$  exchange at equilibrium was measured as a function of the ATP concentration in constant ratio with ADP. In such an experiment the rate should rise to a maximum at high nucleotide levels. However it has been pointed out (63,64) that the formation of dead-end complexes may cause inhibition of exchange rates at high substrate concentration. In view of the observation that ADP may form such



complexes (see Chapter III) the inhibition noted at 1 mM ADP in figure 19 was not unexpected. The subsequent experiments reported here are carried out at ADP concentrations of about 0.2 mM with an ADP:ATP ratio of from 0.1 to 0.2.

The effect of varying the CoA concentration in fixed ratio with  $P_i$  on the rate of ATP  $\leftrightarrow$  ADP exchange is shown in figure 20. Under these conditions the rate rises to a maximum at 30-100  $\mu\text{M}$  CoA but is inhibited as the CoA concentration is increased to higher levels. Nevertheless, the exchange rate does not approach zero even at very high concentrations of CoA as would be predicted from MECHANISM III (figure 18). Furthermore the inhibition observed at a CoA concentration greater than 100  $\mu\text{M}$  is most likely due to formation of dead end complexes between enzyme and CoA, a possibility supported by our observation that CoA concentrations in the range 0.5 mM to 1.0 mM will inhibit the initial velocity assay for SCS.

A similar experiment on the effects of succinate concentration on ATP  $\leftrightarrow$  ADP exchange is shown in figure 21. There appears to be an initial rise to a maximum rate near 1.0 mM succinate, followed by a slight inhibition that levels off when a concentration of 3.0 mM succinate is reached. Further increase in succinate concentration does not significantly inhibit the exchange rate under these conditions. This result is also not in harmony with the sequential scheme (SCHEME A), or the ping-pong reaction (SCHEME B) discussed above.

The results of figure 22 demonstrate that when succinate is varied in constant ratio with succinyl CoA at a low  $P_i$  concentration a distinct inhibition occurs such that the exchange rate approaches zero





Figure 19. Effect of varying ATP in constant ratio with ADP on the rate of  $\text{ATP} \leftrightarrow \text{ADP}$  exchange.  
Each reaction mixture contained at equilibrium in a volume of 1.0 ml, 1.7 mM succinate,  
8.3 mM  $P_i$ , 97  $\mu\text{M}$  CoA, 27  $\mu\text{M}$  succinyl CoA, 10 mM  $\text{MgCl}_2$ , SCS (0.02 units), 50 mM Tris-HCl, 50 mM KCl, pH 7.2, and ADP:ATP in constant ratio as shown.

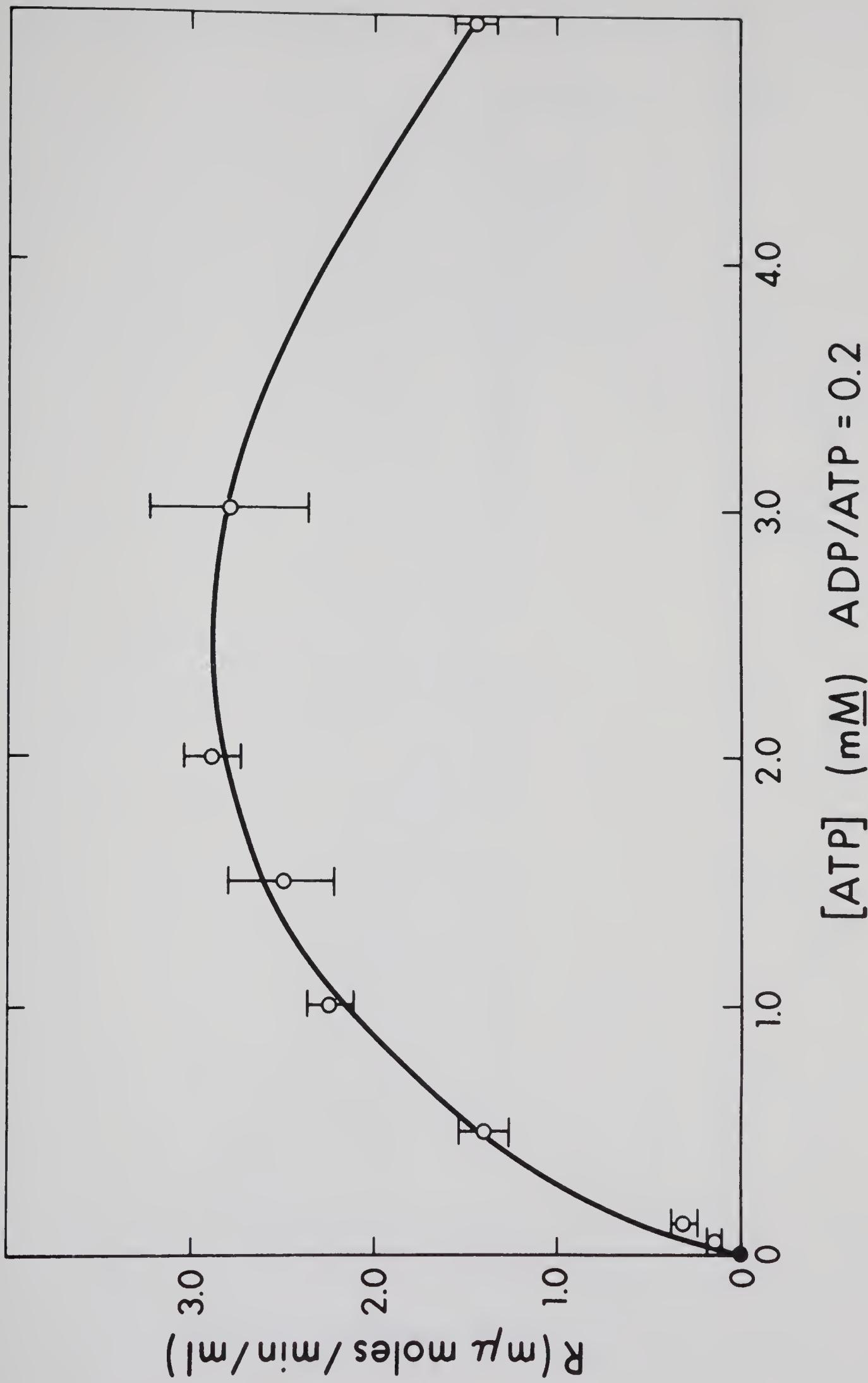






Figure 20. Effect of CoA varied in constant ratio with  $P_i$  on the rate of  $ATP \leftrightarrow ADP$  exchange.  
Each reaction mixture contained at equilibrium in a volume of 1.0 ml, 0.20 mM ADP,  
1.8 mM ATP, 0.16 mM succinate, 80  $\mu$ M succinyl CoA, 10 mM  $MgCl_2$ , SCS (0.02 units),  
50 mM Tris-HCl, 50 mM KCl, pH 7.2, and CoA: $P_i$  in constant ratio as shown.

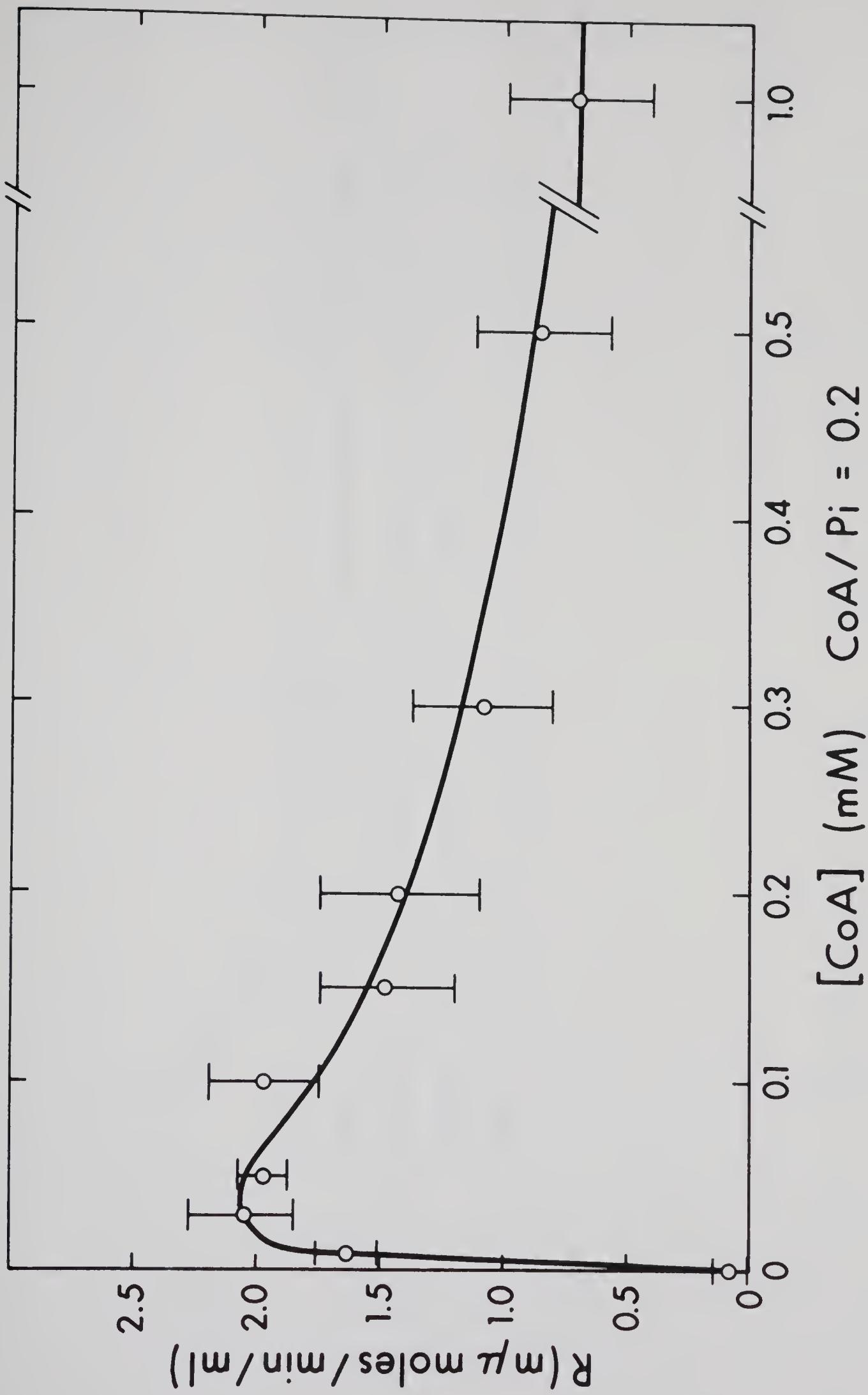






Figure 21. Effect of succinate varied in constant ratio with  $P_i$  on the rate of  $ATP \leftrightarrow ADP$  exchange. Each reaction mixture contained at equilibrium in a volume of 1.0 ml, 0.20 mM ADP, 1.6 mM ATP, 65  $\mu$ M CoA, 25  $\mu$ M succinyl-CoA, 10 mM  $MgCl_2$ , SCS (0.02 units), 50 mM Tris-HCl, 50 mM KCl, pH 7.2, and succinate: $P_i$  in constant ratio as shown.

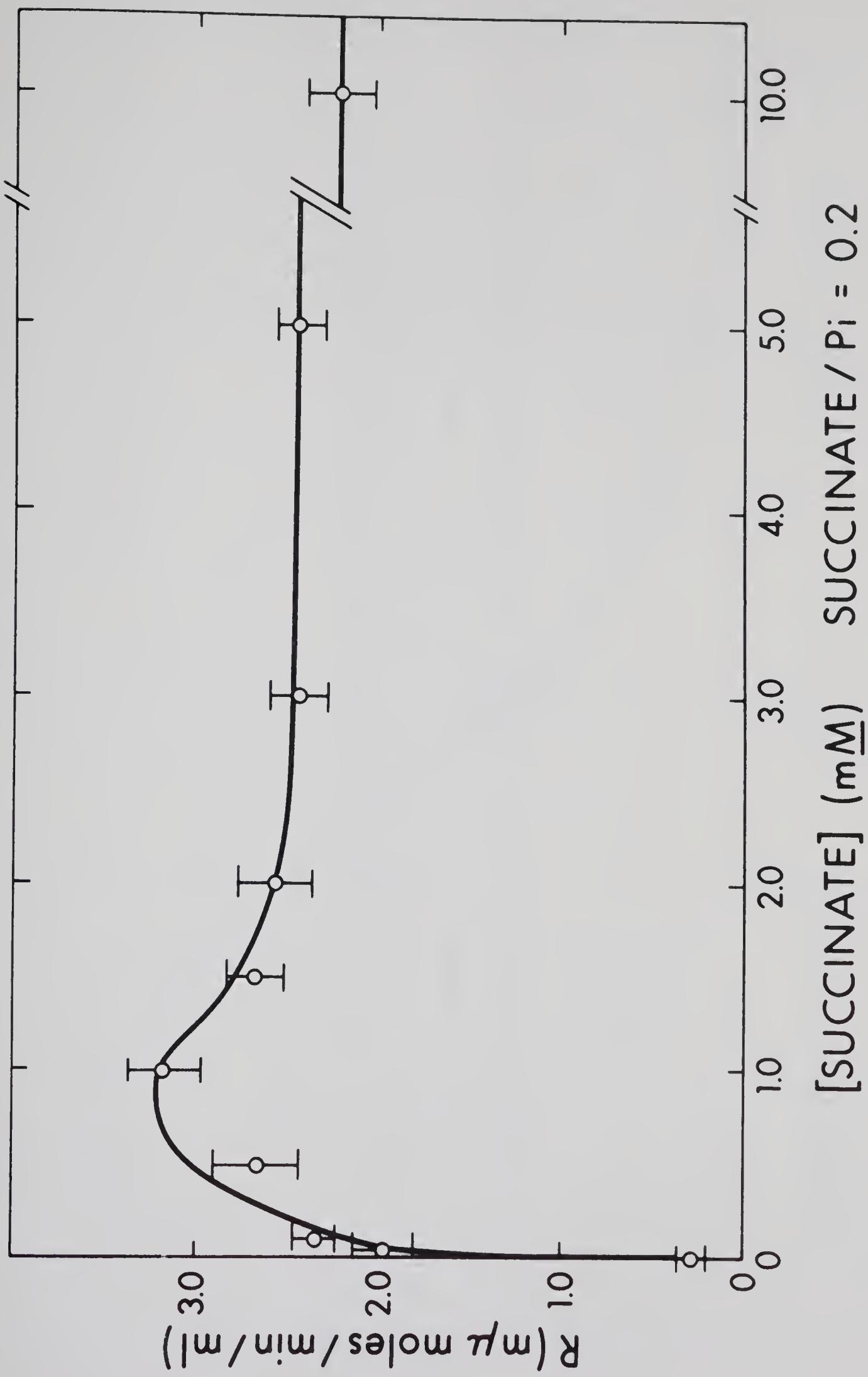
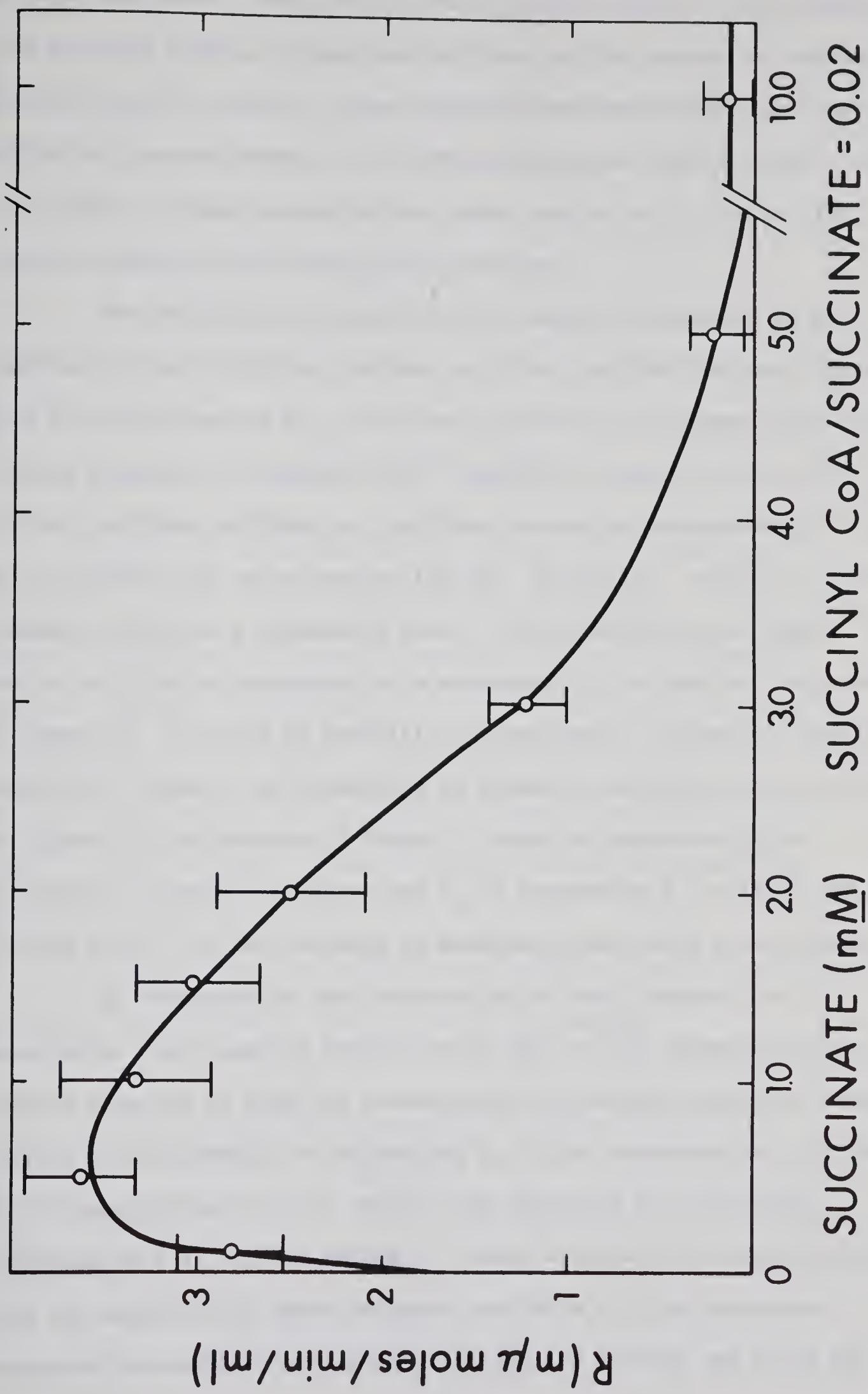






Figure 22. Effect of succinate varied in constant ratio with succinyl CoA on the rate of ATP  $\leftrightarrow$  ADP exchange. Each reaction mixture contained at equilibrium in a volume of 1.0 ml, 0.20 mM ADP, 1.60 mM ATP, 0.10 mM P<sub>i</sub>, 1.0  $\mu$ M CoA, 10 mM MgCl<sub>2</sub>, SCS (0.02 units), 50 mM Tris-HCl, 50 mM KCl, pH 7.2 and succinate: succinyl CoA in constant ratio as shown.





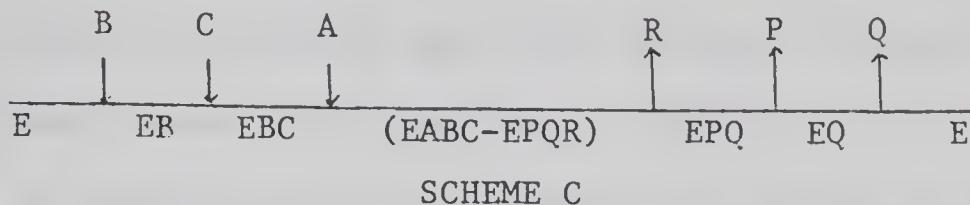
at 10 mM succinate. This could possibly be the result of the formation of a dead-end complex between succinyl CoA and the enzyme, or between succinate and the enzyme. These possibilities seem unlikely as the inhibition is near maximal at 5.0 mM succinate and 100  $\mu$ M succinyl CoA and neither of these concentrations shows inhibition in the initial velocity assays in the appropriate direction.

The inhibition in figure 22 then might be explained by a compulsory order of product release such that succinyl CoA must dissociate from the enzyme before ADP. This result would be in harmony with the binding sequence of MECHANISM III. However in figure 20, where CoA: $P_i$  is varied, succinate is fixed at less than saturating concentration (0.16 mM) while succinyl CoA is saturating (80  $\mu$ M) throughout, and ATP  $\rightleftharpoons$  ADP exchange occurs at a reasonable rate. If the inhibition in figure 22 is due to an effect of succinate as a substrate in the reaction the results of figure 21, in which no inhibition by succinate is observed, must be explained. Since  $P_i$  is saturating in figure 21 and less than saturating in figure 22, the results in figure 21 might be explained by an interaction between the enzyme and  $P_i$  at saturating  $P_i$  to allow for a pathway of ATP  $\rightleftharpoons$  ADP exchange at saturating succinate concentration.

As discussed in the introduction to this chapter, the observation that complete inhibition of ATP  $\rightleftharpoons$  ADP exchange does not occur in this reaction at high CoA concentration or at high succinate concentration in the presence of saturating  $P_i$  is not consistent with MECHANISM III (figure 18) nor does it reflect the operation of a ping-pong mechanism such as that of SCHEME B. These results can be rationalized with the existence of minor pathways available at high substrate concentration which do not involve initial ATP binding and final ADP



dissociation from the enzyme. Two such pathways are possible as variations of SCHEME A with altered order of substrate addition and product release. Retaining the same letter identification of substrates and products as in SCHEME A, the first of these could be written:

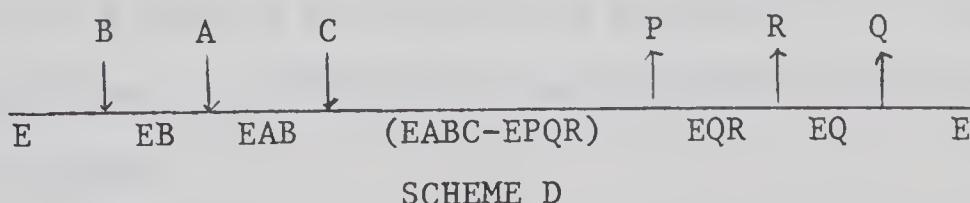


an equation for  $V^*$ , the rate of  $A \rightleftharpoons R$  ( $ATP \rightleftharpoons ADP$ ) exchange can be written:

$$V^* = \frac{K_5 K_7 A^* (EBC)}{K_6 + K_7} \quad [3]$$

$A^*$  is concentration of labelled A and  $K_5$ ,  $K_6$ ,  $K_7$  are again rate constants. (EBC) is the concentration of EBC complex. This is obviously identical in form with equation [2] for the ping-pong reaction. However in this case the rate is proportional to the concentration of the interior complex EBC which will rise to a maximum as any one of the non-labelled substrate-product pairs is raised towards infinity. Thus no substrate inhibition would be observed for this pathway.

A second possible variation of SCHEME A involves ATP and ADP as the second substrate-product pair in the sequence:



Again the same letter identification as used in SCHEME A is retained.

The equation for this pathway then is:

$$V^* = \frac{K_3 K_5 K_7 K_9 A^* C (EB)}{K_4 K_6 K_9 + K_4 K_7 K_9 + K_5 K_7 K_9 C + K_4 K_6 K_8 P} \quad [4]$$



Again  $V^*$  is the rate of appearance of labelled R from labelled A,  $A^*$  is the concentration of labelled A, K's are rate constants and (EB) is the concentration of the EB complex. This scheme predicts that the interior substrate-product pair (C,P) will inhibit  $A \rightleftharpoons R$  ( $ATP \rightleftharpoons ADP$ ) exchange while the exterior pair (B,Q) will not. This is in conformity with Cleland's general rule considered earlier.

It should be noted that substrate inhibition occurs if either member of substrate-product pair combines between the points of addition and release of the exchanging pair. Thus other possible pathways where ATP and ADP addition and dissociation is non-symmetrical will not constitute different pathways for the purposes of predicting inhibition patterns.

The above analysis shows that complete substrate inhibition will not be observed whenever a pathway exists such that the varied substrate, succinate or CoA, can add before the exchanging substrate, ATP, and ADP, the exchanging product can dissociate before the variable product, succinyl CoA or  $P_i$ . This would suggest that no pathway such as SCHEME C is available in figure 22 where succinate varied in ratio with succinyl CoA causes nearly complete inhibition of exchange. Schemes A, B, or D could explain these results but the failure of saturating succinate in figure 21 and saturating succinyl CoA in figure 20 to cause inhibition is difficult to reconcile with any compulsory binding sequence.



The isotope exchange results then demonstrate that reaction sequences initiated by succinate or CoA binding to the free enzyme are possible for SCS. It would be emphasized that all of the pathways discussed above are detected only by the sensitive means of isotope exchange kinetics. They are therefore likely to be minor pathways operable only under certain extremes of reactant concentration. The major catalytic route is probably the partially random sequential pathway of MECHANISM III (figure 18) proposed on the basis of initial rate kinetic studies. The isotope exchange results serve to point out that this is not an exclusive pathway and that minor routes are permitted.

In all of these experiments, ATP and ADP are observed to undergo a slow isotope exchange in the absence of other substrates, which is stimulated by the addition of these substrates. This slow ATP  $\longleftrightarrow$  ADP exchange which is independent of the presence of other substrates indicates that the ping-pong mechanism with ADP dissociation preceding addition of succinate and/or CoA, is not forbidden. Thus the complete kinetic mechanism must include a large number of alternative minor pathways.

The explanation of the difference between the succinate effect on ATP  $\longleftrightarrow$  ADP exchange in the experiments of figures 21 and 22 on the basis of a specific interaction between enzyme and  $P_i$  is presently hypothetical. The result may yet be explained on the



basis of dead-end complex formation or possibly a general ion effect as  $P_i$  reaches a concentration of 50 mM in the experiment of figure 21. Further exchange studies at various combinations of reactant concentrations may shed some light on this matter but such experiments are technically difficult and complicated in interpretation due to the tendency of this enzyme to form dead-end complexes with its substrates. If the result is indeed a specific effect of  $P_i$  binding in overcoming substrate inhibition by succinate then it might possibly be mediated by a conformational change in the enzyme. In the next chapter evidence is presented that a conformational change does occur upon binding of  $P_i$  to SCS as well as upon phosphorylation of the active site histidine residue.

### C. Summary

Studies of the kinetics of ATP  $\leftrightarrow$  ADP isotope exchange show that the partially random sequential kinetic mechanism (figure 18) proposed on the basis of initial rate kinetic data is not an exclusive pathway. ATP  $\leftrightarrow$  ADP isotope exchange patterns did not show complete substrate inhibition when CoA: $P_i$  was varied in the presence of saturating succinyl CoA or when succinate: $P_i$  was varied. Nearly complete inhibition was observed when succinate:succinyl CoA was varied.



These results can be interpreted in terms of a wide variety of minor pathways of substrate binding and product release available to the enzyme under various conditions.



## CHAPTER V

## TRYPSIN-CATALYZED INACTIVATION OF SUCCINYL CoA SYNTHETASE

A. Introduction

The interaction between SCS and its substrates is clearly complex. The observations of substrate synergism (14) show that substrates may stimulate the rate of partial reactions in which they have no direct role, and the presence of all substrates results in a rate for the overall reaction that is much greater than that for any isolated partial reaction. The kinetics of the reaction suggest that it proceeds through a quaternary complex, involving the binding of all substrates before the dissociation of any products, despite the participation of a phosphoryl-enzyme covalent intermediate in the catalysis. These observations are consistent with the induced fit concept of Koshland (65) in that the interaction of all substrates with the enzyme would induce an optimal conformation for reaction at the active site.

The discovery that  $P_i$  apparently interacts with the enzyme in such a way that alternate reaction sequences are available at high substrate concentration (see Chapter IV) also suggests the possibility of specific conformational changes as a result of the binding of this ligand. Although this result is only tentative, evidence that a conformational change accompanies  $P_i$  binding would provide at least circumstantial support for the concept.



The subunit composition data of Bridger (43) can most easily be reconciled with an  $\alpha_2\beta_2$  type of subunit structure. His observation that only the smaller of the two apparent subunit types is phosphorylated suggests that there are two active sites in the catalytic molecule of 140,000 M.W. However measurements of the stoichiometry of phosphorylation by either ATP or succinyl CoA + P<sub>i</sub> leads to values of 1.0 to 1.5 phosphoryl residues per 140,000 M.W. (12, 35, 42). Table II shows data for phosphorylation of the enzyme used in this study. Assuming that two active sites do exist in the native molecule, the degree of phosphorylation may reflect a conformational change on phosphorylation resulting in an anti-co-operative effect between the two active sites of the type recently described by Levitzki *et al.* (44). The data of Table II also confirm the observations of Ramaley *et al.* (12) that apparently homogeneous enzyme preparations, exhibiting a range of specific activities, all accept approximately one phosphoryl group.

All of these observations are consistent with the possibility of specific conformational alterations in SCS caused by the binding of substrates. The effect of substrates on the rate of trypsin-catalyzed inactivation of succinyl CoA synthetase was examined in an attempt to detect these conformational alterations.

#### B. Results and Discussion

The results of a preliminary investigation of the effect of substrates on the trypsin catalyzed inactivation of SCS are shown in Figure 23A. ATP is seen to protect the enzyme, while succinate enhances trypsin sensitivity. There is only a small effect of CoA while the presence of all substrates significantly enhances sensitivity



to trypsin. The control is observed to give a biphasic curve on the first order plot of figure 23A. This plot suggests the possibility of two forms of SCS in the preparation with differential sensitivity to trypsin. The enzyme preparation used in this experiment was not dephosphorylated prior to use and it appeared possible that phosphorylation might be the source of this differential sensitivity to trypsin displayed by the enzyme in the absence of substrates. This possibility was further supported by the consideration that ATP is known to phosphorylate SCS (12) while succinate is capable of dephosphorylating the enzyme (25). Thus the degree of phosphorylation of the enzyme might explain all the results of figure 23A.

The data of figure 23B represents the results of an investigation of the effect of phosphorylation of SCS on its susceptibility to trypsin inactivation. The P/E ratios represent phosphate residues per 140,000 M.W. First consider enzyme with a P/E ratio of zero which corresponds to enzyme that has been dephosphorylated prior to the experiment. When this enzyme is treated with trypsin a linear first order plot results. The pseudo-first order rate constant for this inactivation,  $k_1$ , is  $0.12 \text{ min.}^{-1}$ . Enzyme which has a P/E ratio of 1.0 also is inactivated in a first order manner and the slope of this line gives a pseudo-first order rate constant,  $k_2$ , of  $0.0014 \text{ min.}^{-1}$ , about one hundred times less than that of the dephosphorylated enzyme. Enzyme with intermediate degrees of phosphorylation gives biphasic first order plots of inactivation. Ray and Koshland (66) point out, in a theoretical analysis of inactivation kinetics, that such curves can



TABLE II

## Stoichiometry of Phosphorylation of SCS

Specific Activity	Phosphoryl Groups/140,000 M.W.
20.5	1.08
23.6	0.88
31.9	1.05
31.0 <sup>1</sup>	1.00

Reactions contained 0.2 mgm SCS, 100  $\mu$ M ATP- $\gamma$ - $^{32}$ P ( $10^8$  cpm/ $\mu$ mole), 10 mM MgCl<sub>2</sub>, 50 mM Tris-HCl, 50 mM KCl, pH 7.2, in a 1 ml. volume.

<sup>1</sup> 10 mM succinyl CoA plus 10 mM  $^{32}$ P<sub>i</sub> ( $10^7$  cpm/ $\mu$ mole) replaced ATP- $\gamma$ - $^{32}$ P. Reactions were incubated for 10 minutes at 25°C and protein bound  $^{32}$ P was assayed as described under experimental procedures (Chapter II).





Figure 23A. Effect of trypsin on the activity of SCS in the presence and absence of substrates.

Reactions contained 0.27 mgm/ml SCS (S.A.=5.2), 50 mM Tris-HCl, 50 mM KCl,  
pH 7.2, 10 mM MgCl<sub>2</sub>, 0.0004% trypsin and other additions as shown.

Concentrations of added substrates: succinate, 10 mM; ATP, 0.5 mM; CoA, 0.2 mM.

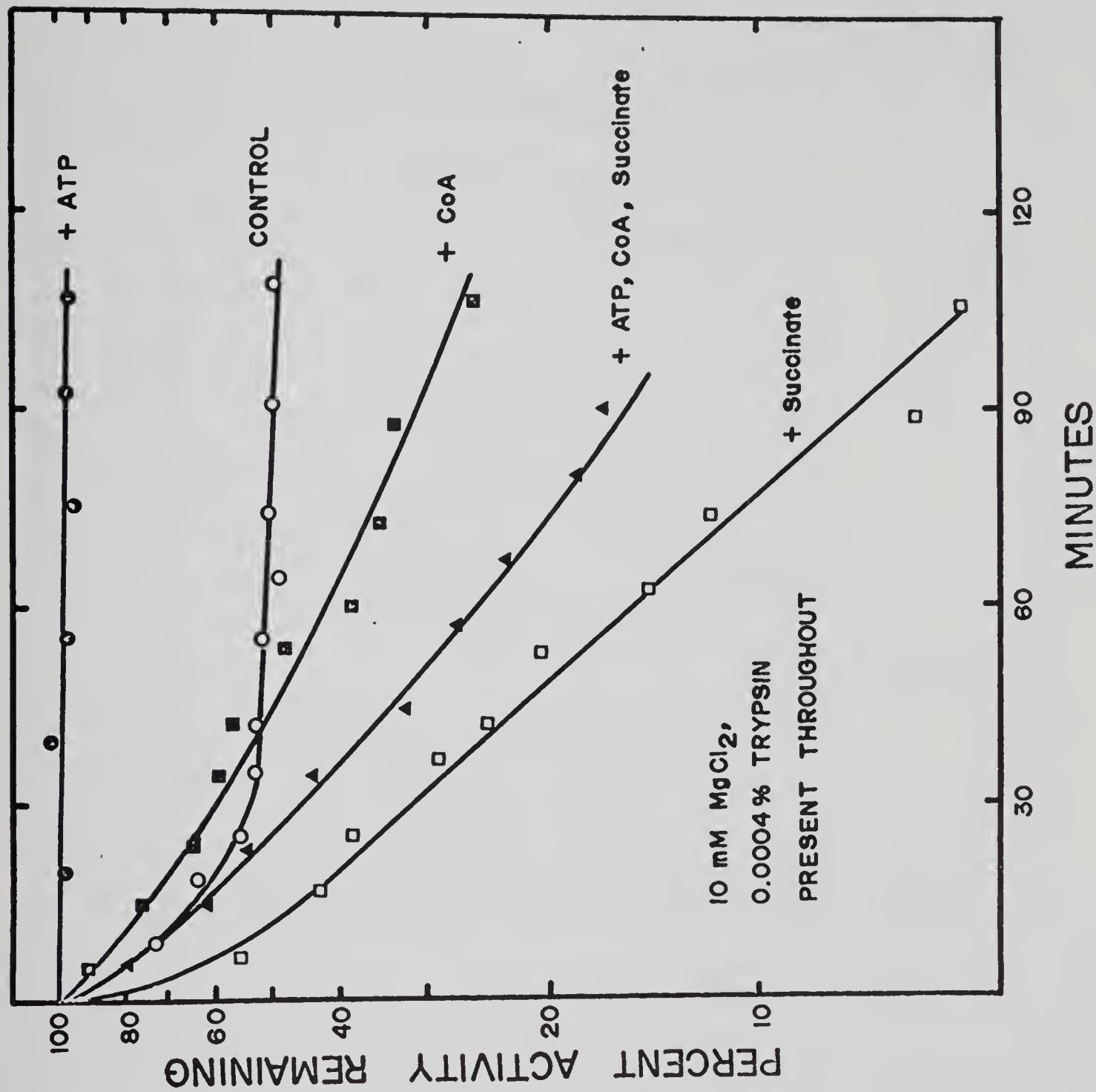
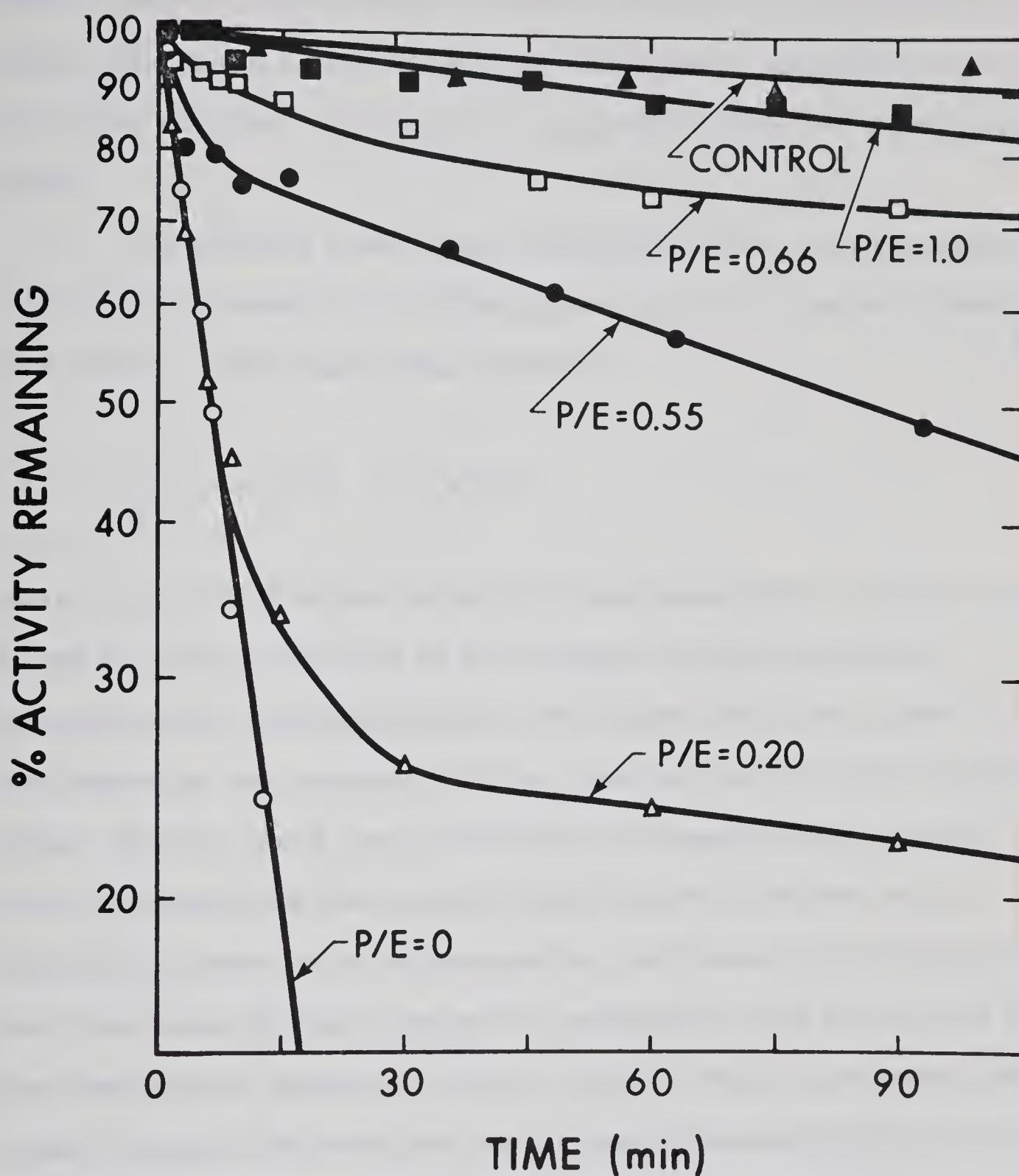






Figure 23B. Effect of phosphorylation of SCS on the rate of trypsin-catalyzed inactivation. Each reaction mixture contained in a volume of 1.02 ml, 50 mM Tris-HCl, 50 mM KCl, pH 7.2, 0.01 mgm/ml trypsin-TPCK, and SCS as follows: ▲ —▲, 0.12 mgm/ml (S.A. = 16.5), trypsin omitted; ■ —■, 0.25 mgm/ml (S.A. = 31.9); □ —□, 0.12 mgm/ml (S.A. = 16.5); ● —●, 0.21 mgm/ml (S.A. = 18.2); Δ —Δ, 0.25 mgm/ml (S.A. = 15.9); ○ —○, 0.20 mgm/ml (S.A. = 7.2). Partially phosphorylated enzyme was prepared by incubating dephospho-enzyme + ATP + EDTA for appropriate times. The reaction mixture was then added directly to a G-50 Sephadex column.





arise from the existence of two forms of the enzyme, active in the assay system, that are inactivated at different rates. In the case of SCS, it appears that there is a difference of two orders of magnitude in the rate of trypsin catalyzed inactivation of dephospho-enzyme and phosphorylated enzyme.

An equation given by Ray and Koshland (67) for the biphasic modification of amino acid residues can be applied to activity loss in this system. This equation has the form:

$$\frac{A}{A_0} = f_1 e^{-k_1 t} + f_2 e^{-k_2 t}$$

where  $A/A_0$  is the fraction of activity remaining after reaction time  $t$ ;  $f_1$  and  $f_2$  are the fractions of the original activity present as dephosphorylated and phosphorylated SCS respectively, and  $k_1$  and  $k_2$  are the respective rate constants for the inactivation of the two enzyme forms. Using  $k_1$  and  $k_2$  as given above, and assuming that the P/E ratio represents the fraction of original activity present as E-P theoretical curves can be calculated for the biphasic plots of figure 23B. Such lines have the same form as the experimental data but are not in good quantitative agreement with the results. This is not surprising as many studies have shown that the degree of phosphorylation of SCS does not correlate in a simple manner with specific activity (12, 42, also see Table II). It is probable that some enzyme that is inactive in the overall assay is still capable of undergoing phosphorylation and thus the assumption that the P/E ratio represents the fraction of active enzyme in the E-P form is not valid.



Nevertheless, the results are in qualitative agreement with this analysis and the above equation will describe the data with a proper choice of  $f_1$  and  $f_2$  values. It should be noted that the specific activities of enzyme used in the experiments of figure 23B varies due to the lability of the dephosphorylated enzyme and the various pretreatments required. It was determined in other experiments that specific activity has no effect on the form of the inactivation plot or the rate constant for inactivation of dephospho-SCS over a range from 5 units per mgm to 25 units per mgm. The results of figure 23B thus support the concept that phosphorylation of SCS strongly protects the enzyme from trypsin-catalyzed inactivation.

ATP protection of dephospho-SCS from trypsin-catalyzed inactivation in figure 23A is thus likely due to phosphorylation of the enzyme. ATP was observed to give a similar protection in other experiments in the presence of EDTA and absence of added  $Mg^{++}$ . A study of phosphorylation of SCS in the presence of ATP and EDTA is shown in figure 24. The results indicate a fairly rapid phosphorylation of the enzyme in the absence of  $Mg^{++}$ . Although the rate of phosphorylation is several orders of magnitude slower than that in the presence of  $Mg^{++}$  (14) it is still rapid enough to account for the protection observed in trypsin-catalyzed inactivation experiments. Table III presents further data on the ATP effect on the inactivation reaction. ATP is found to have no further effect on previously phosphorylated enzyme. ADP at relatively high concentration gives slight protection to the dephosphorylated enzyme, while the ATP analogue  $\beta$ - $\gamma$ -methylene ATP (Aopopcp) has no effect. This analogue is inhibitory in the initial velocity assay at equimolar concentrations with ATP which indicates that it can bind to the enzyme.



These results suggest that the protection from trypsin is a property of the phosphorylated enzyme only and that the protection may be due to a conformational change in the protein molecule rather than a shielding of a sensitive residue at the active site.

It has been observed previously that succinate will dephosphorylate succinyl CoA synthetase in the presence of  $Mg^{++}$  (25,31). Figure 25 illustrates this effect which we find to be somewhat more rapid at lower concentration of succinate than indicated by the results reported by Robinson *et al.* (25). It was also noted that no appreciable dephosphorylation occurred in the presence of EDTA and absence of added  $Mg^{++}$ .

The effect of succinate on the trypsin catalyzed inactivation reaction is shown in figure 26. Each sample was incubated for 120 minutes with the indicated succinate concentrations before trypsin addition. Assays were carried for 90 minutes after trypsin addition, and at the end of this assay period the P/E ratios shown in the figure were determined. The results indicate that succinate caused a dephosphorylation of the enzyme and that the degree of dephosphorylation is reflected in increased sensitivity to trypsin. Also, no dephosphorylation, and no increase in sensitivity occurred in the presence of succinate + EDTA. These results are all in accord with the dephosphorylation effects of succinate as depicted in figure 25 and it appears that changes in trypsin-catalyzed inactivation rates can in all cases be correlated to the degree of phosphorylation of SCS. The data of table III indicates that succinate has very little effect on the inactivation of dephosphorylated enzyme.





Figure 24. Phosphorylation of SCS by ATP- $\gamma$ - $^{32}$ P in the presence of EDTA and absence of added Mg<sup>++</sup>. Each reaction mixture contained in a volume of 3.1 ml, 0.37 mgm/ml SCS (S.A. 17.5), 10 mM EDTA, 50 mM Tris-HCl, 50 mM KC1, pH 7.2, and ATP- $\gamma$ - $^{32}$ P ( $10^8$  cpm/ $\mu$ mole) as shown. 200  $\mu$ l samples were withdrawn at the times indicated and assayed as described under experimental procedures (Chapter II).

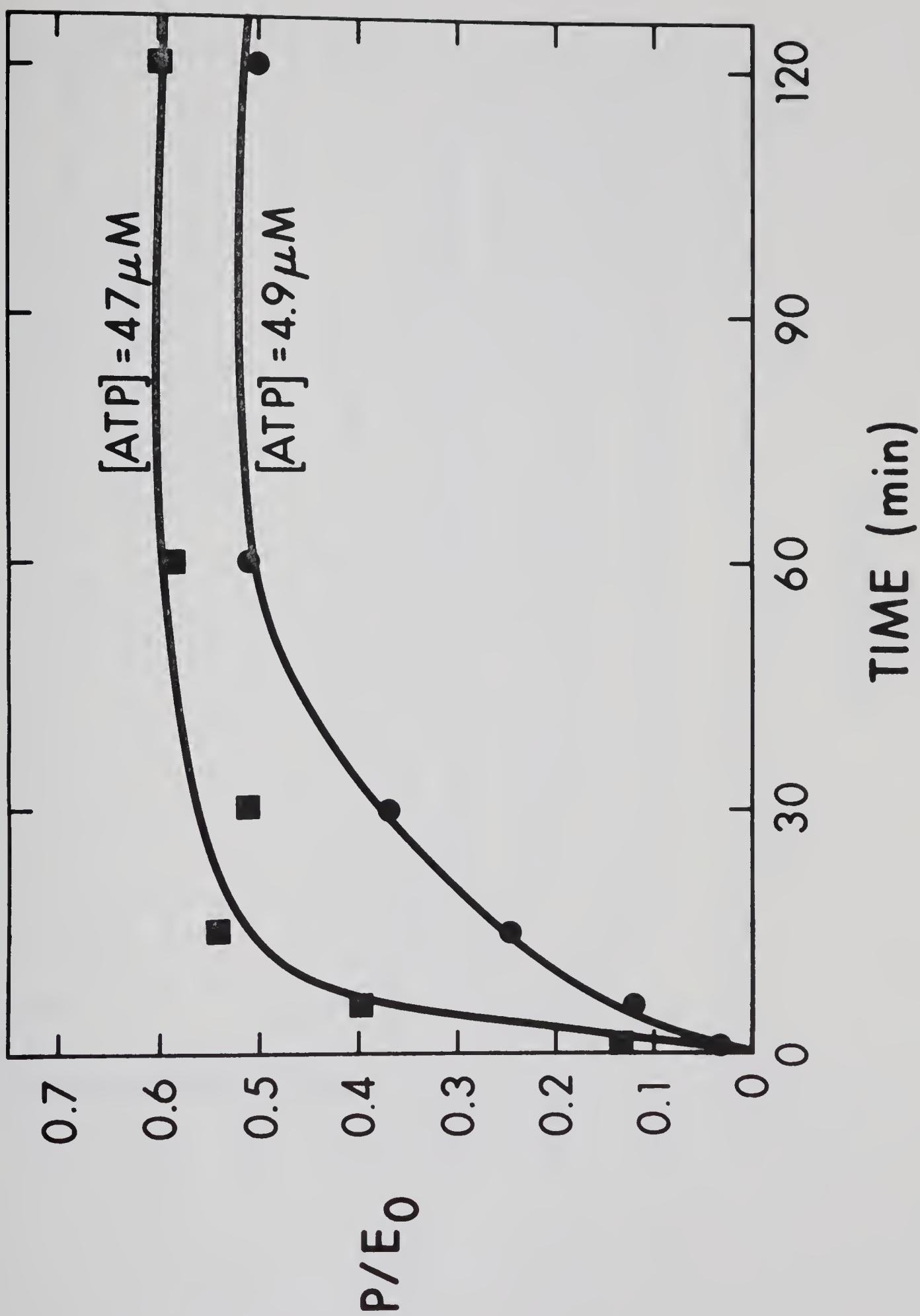






Figure 25. Dephosphorylation of  $^{32}\text{P}_i$  labelled SCS, in the presence of succinate +  $\text{Mg}^{++}$ .  
Each reaction mixture contained in a volume of 3.4 ml, 0.37 mgm/ml SCS  
(S.A. = 20.3, P/E = 0.73), 50 mM Tris-HCl, 50 mM KCl, pH 7.2, and succinate and  
 $\text{MgCl}_2$  as shown. 200  $\mu\text{l}$  samples were withdrawn at the times indicated and  
assayed as described under experimental procedures (Chapter II).

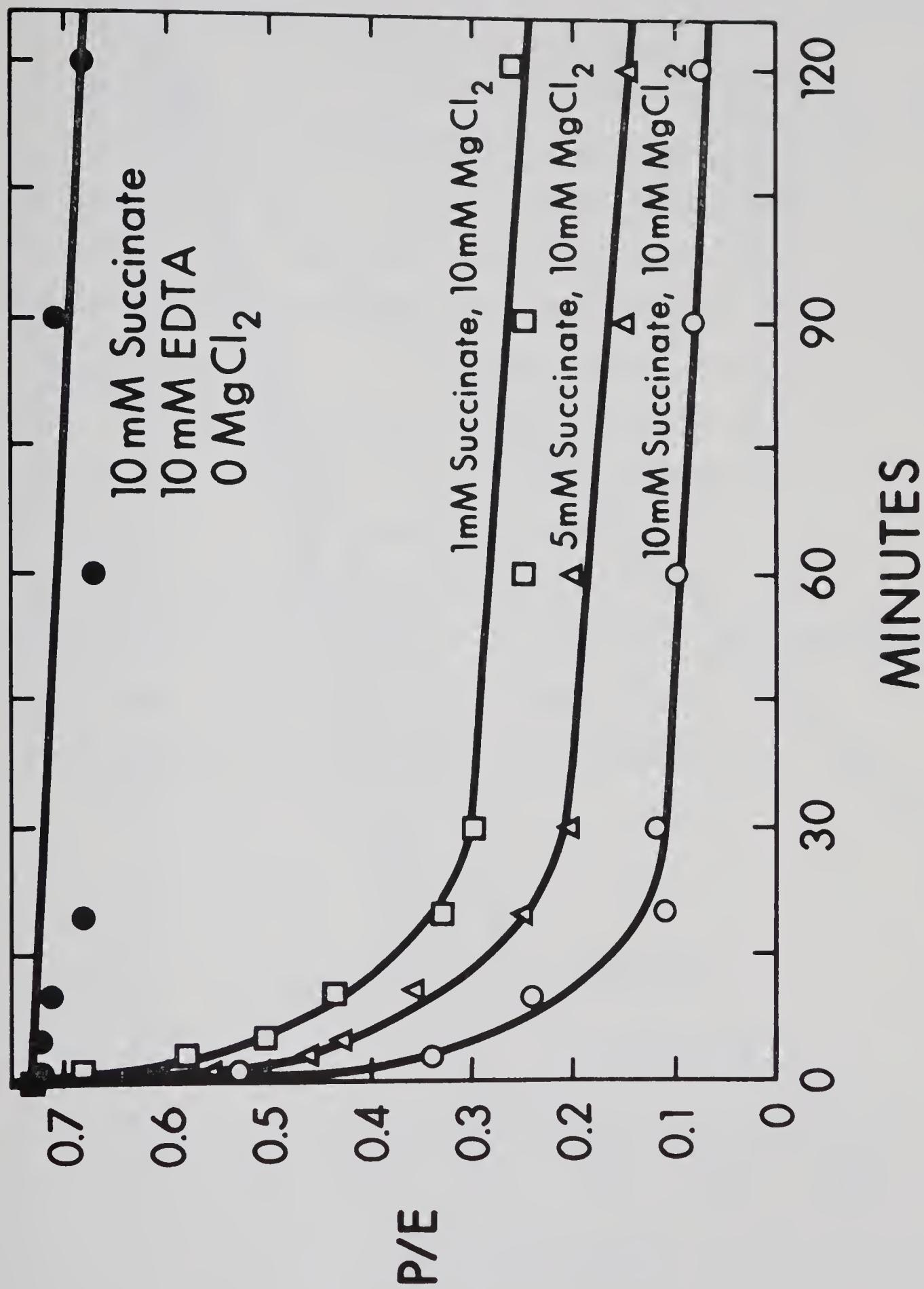






Figure 26. Effect of succinate on trypsin catalyzed inactivation of SCS. The reaction mixtures contained in a volume of 1.16 ml, 0.25 mgm/ml SCS (S.A. = 19.7, P/E = 0.64), 10 mM MgCl<sub>2</sub>, 0.01 mgm/ml trypsin-TPCK, 50 mM Tris-HCl, 50 mM KCl, pH 7.2 with the following additions and/or omissions:  
■ — ■, trypsin omitted; ○ — ○, none; ▲ — ▲,  
10 mM succinate, 10 mM EDTA added, MgCl<sub>2</sub> omitted; ○ — ○,  
1 mM succinate added; ● — ●, 5 mM succinate added;  
Δ — Δ, 10 mM succinate added; □ — □, 0.76 mgm/ml  
(S.A. = 11.7) dephospho-SCS added, phosphorylated SCS  
omitted. Reactions were incubated at 25°C for 120 minutes  
before trypsin addition. P/E ratios shown in the figure  
were determined after 90 minutes of trypsin digestion.

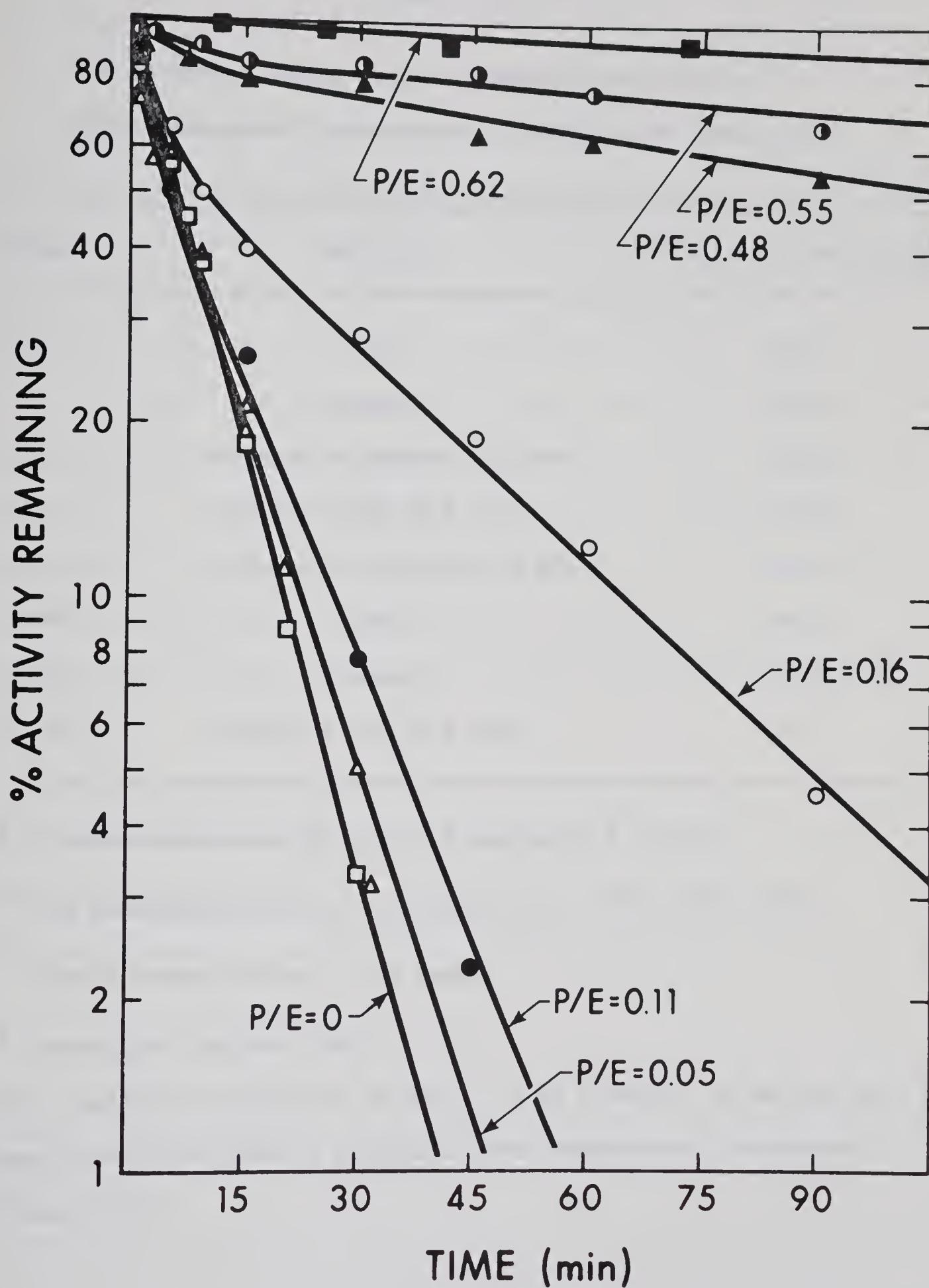




TABLE III

Effect of Substrates and Substrate Analogues on  
 Trypsin-Catalyzed Inactivation of Succinyl CoA Synthetase

Enzyme	Addition	% Activity Remaining <sup>4</sup>
E <sup>1</sup>	none	98.1
E	trypsin	15.9
E	trypsin <sup>3</sup> + AOPOPCP (0.4 mM)	14.2
E	trypsin + ADP (0.2 mM)	31.8
E	trypsin + succinate (10 mM)	18.5
E-P <sup>2</sup>	none	96.8
E-P	trypsin	91.6
E-P	trypsin + ATP (0.1 mM)	91.2

<sup>1</sup> E: dephosphorylated SCS (0.6-0.8 mgm/ml; S.A. = 25)

<sup>2</sup> E-P: phosphorylated SCS (0.33 mg/ml; S.A. = 22; P/E = 0.75)

<sup>3</sup> Trypsin concentration: 0.016 mgm/ml

<sup>4</sup> Incubation time was 9 min.

All reactions contained 10 mM MgCl<sub>2</sub>, 50 mM Tris-HCl, 50 mM KCl, pH 7.2.

Assays were performed as described under experimental procedures  
 (Chapter II).



The ability of inorganic phosphate to protect the enzyme from inactivation by trypsin is demonstrated in figure 27. This effect occurs at  $P_i$  concentrations in the range of  $K_m$  and most likely reflects binding at the active site. The experiments of figures 23B and 27 are done at the same trypsin concentration and the pseudo-first-order rate constant for inactivation of the dephospho-enzyme in figure 27 is  $0.13 \text{ min.}^{-1}$  in good agreement with that obtained from figure 23B. The rate constant for inactivation in the presence of 50 mM  $P_i$  is  $0.012 \text{ min.}^{-1}$  which is an order of magnitude faster than that found for enzyme phosphorylated with 1.0 moles of  $P_i$  per mole of enzyme (figure 23). The available active sites would be expected to be saturated with  $P_i$  at a level of 50 mM, and the results suggest that the protective effect of  $P_i$  binding is quantitatively and/or qualitatively different from that of covalent phosphorylation at the active site.

The data of figure 27 can be used to obtain rate constants for inactivation at various  $P_i$  concentrations. Such rate constants can be used in equation [13a] of O'Sullivan and Cohn (68) to obtain a value of the dissociation constant for  $P_i$ . This equation has the form:

$$\frac{k'}{k_1} = \frac{K}{[P_i]} \left( 1 - \frac{k'}{k_1} \right) + \frac{k_2}{k_1}$$

where  $k'$  is the apparent first order rate constant for inactivation in the presence of  $P_i$  and  $k_1$  is the apparent first order rate constant for inactivation of controls in the absence of  $P_i$ .  $K$  is the dissociation constant for  $P_i$ . A plot of  $k'/k_1$  vs  $(1 - (k'/k_1)) / [P_i]$  is shown in figure 28. The slope gives a value for the dissociation constant for  $P_i$ .





Figure 27. Effect of  $P_i$  on trypsin-catalyzed inactivation of SCS.

Each reaction contained in a volume of 1.09 ml, 0.420 mgm/ml SCS (S.A. = 22.2), 0.01 mgm/ml trypsin-TPCK, 10 mM  $MgCl_2$ , 50 mM Tris-HCl, 50 mM KCl, pH 7.2 and  $P_i$  as follows: • —•, none; ▲ —▲, none, trypsin omitted; Δ —Δ, 2 mM; □ —□, 5 mM; ■ —■, 10 mM; ○ —○, 25 mM; ◊ —◊, 50 mM.

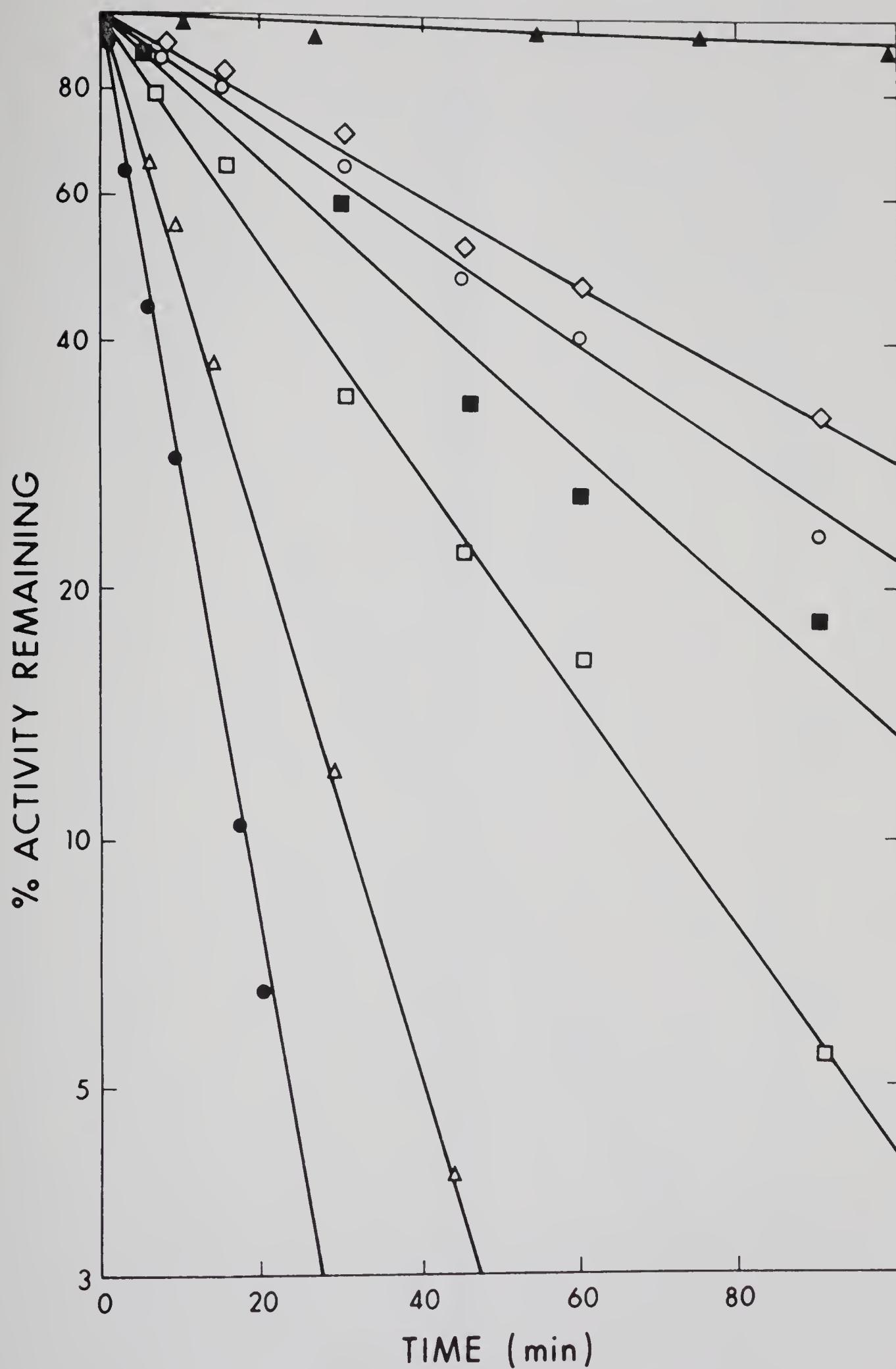
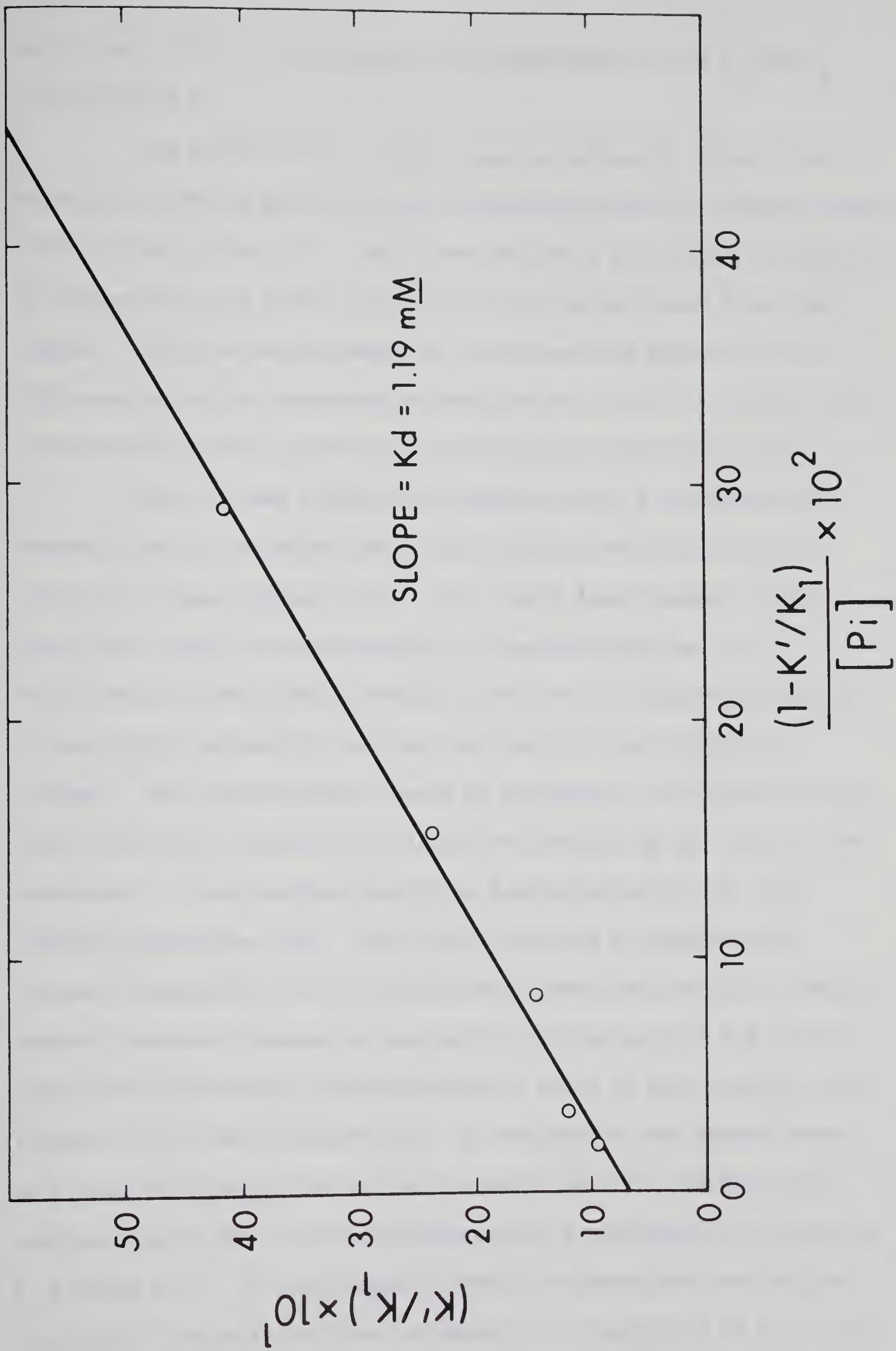






Figure 28. Plot of equation [13a] of O'Sullivan and Cohn (68) using data derived from figure 27. See text for details. Abcissa units are  $1/\text{mM}$ .





of 1.2 mM. This is of the same order of magnitude as the  $K_m$  for  $P_i$ : 2.6 mM (Table I).

The half-times of activity loss on storage in 50 mM Tris-Cl, 50 mM KCl at pH 7.2 and 4°C for both dephosphorylated and phosphorylated SCS are given in Table IV. The values reflect a much greater instability of dephosphorylated enzyme relative to the phosphorylated form, and suggest a possible rearrangement of the enzyme upon phosphorylation. This observation has considerable practical value since it implies that the preferred form for storage is the fully phosphorylated form.

All of these results are consistent with a conformational change in the SCS molecule upon phosphorylation of the active site histidine or upon binding of  $P_i$ . This result lends support to the possibility that the stoichiometry of phosphorylation may be reconciled with the apparent subunit structure by considering negative co-operativity between two active sites subunits per 140,000 M.W. tetramer. Such an interaction would be analogous to the "half-of-the-sites reactivity" phenomenon described by Levitzki et al. (44) for CTP synthetase. A very similar result has been obtained with E. coli alkaline phosphatase (69). This enzyme involves a phosphoserine covalent intermediate and has been shown to have two identical subunits. However phosphate labeling of the active site serine (70) and "active sites burst titrations" (71) have led to a value of approximately one phosphate per dimer of 86,000 M.W. In the case of this enzyme there is strong evidence for two active sites with different dissociation constants for  $P_i$  (69) as well as evidence for a conformational change on  $P_i$  binding (72). In this regard it should be emphasized that in the case of SCS, there is no direct evidence of the existence of two active



TABLE IV  
Stability of SCS in Tris-Cl Buffer

Enzyme	Half-time of Inactivation (days)
dephosphorylated	1.2
phosphorylated	99.6

Buffer was 50 mM Tris-Cl, 50 mM KC1, pH 7.2. Solutions were stored at 4°C. Concentration of dephosphorylated enzyme was 0.6 mgm/ml. Concentration of phosphorylated enzyme was 0.6 mgm/ml (P/E = 1.0).



sites per 140,000 M.W. unit. The hypothesis is based entirely on the existence of two types of subunits of sizes such that an  $\alpha_2\beta_2$  structure appears likely. It remains possible that the subunits which appear identical on SDS-polyacrylamide-gel electrophoresis are in fact very slightly different such that only one active site per tetramer exists, or that non-symmetrical association of identical subunits leads to non-identical active site regions in the tetramer.

Nevertheless the existence of a conformational change on phosphate binding is consistent with the possibility of negative co-operativity between identical active sites, and comparison with the very similar results for alkaline phosphatase at least lends precedent to the concept. A specific effect of phosphate on enzyme conformation is also in accord with its apparent ability to stimulate ATP — ADP exchange at high succinate concentration (Chapter IV).

No direct effect of substrates other than  $P_i$  on the trypsin-catalyzed inactivation of SCS has been noted. The effects of succinate and ATP in figure 23A can be explained by their effects on phosphorylation of the enzyme and the small effect of ADP at very high concentration (Table III) is of doubtful significance. The synergistic effect of substrates may possibly be explained by favourable juxtaposition of various reactive groups in a quaternary complex without requiring a change in enzyme conformation. However it has been pointed out (73) that very small changes in the active site conformation of an enzyme may have large effects on rates of catalysis. It is therefore possible that small changes in enzyme arrangement at the active site of SCS, induced by substrates and



resulting in substrate synergism, may not be detected using trypsin sensitivity as a probe of enzyme conformation.

The effects of ATP and succinate on phosphorylation of the enzyme are interesting. They imply that the  $Mg^{++}$  complex of ATP is not absolutely required for binding to this enzyme, although it is greatly preferred; in addition they indicate a required role for  $Mg^{++}$  in the dephosphorylation reaction to form succinyl CoA and  $P_i$ .

### C. Summary

Succinyl CoA synthetase is strongly protected from trypsin catalyzed inactivation by phosphorylation of the active site histidine residue. Pseudo-first order rate constants for inactivation of dephospho-enzyme and phosphorylated enzyme are  $0.12\text{ min}^{-1}$  and  $0.0014\text{ min}^{-1}$ , respectively. Binding of inorganic  $P_i$  to the enzyme also results in protection, but is less effective than phosphorylation even at saturating  $P_i$  concentration. Aopopcp, ADP, and succinate have no appreciable effect on the trypsin sensitivity of dephosphorylated enzyme. ATP is found to phosphorylate SCS in the presence of EDTA and absence of added  $Mg^{++}$  while succinate is observed to promote the dephosphorylation of the enzyme only in the presence of  $Mg^{++}$ .

The results can be interpreted in terms of a conformational change induced by active site phosphorylation. Such a conformational change might result in an apparent negative co-operativity between two active sites in the catalytic 140,000 M.W. tetrameric form of the enzyme.



## CHAPTER VI

## SUBSTRATE BINDING STUDIES

A. Introduction

Differences between the dissociation constants of substrates from the free enzyme and their Michaelis constants in the overall reaction might be an explanation for a preferred pathway in a random mechanism. Initial rate kinetic studies of SCS are consistent with a partially random sequential binding sequence involving the initial combination of ATP or ADP with the free enzyme (figure 18). The kinetics of  $\text{ATP} \rightleftharpoons \text{ADP}$  isotope exchange (Chapter IV) suggest that other pathways are possible at high concentrations of specific substrates. Thus, it appears that an overall completely random ter-ter mechanism (38) with a preferred pathway may describe the kinetic mechanism of this enzyme. In this case, all substrates would be expected to bind to the free enzyme and a comparison of Michaelis and dissociation constants should be possible.

The apparent subunit structure of this enzyme (43) suggests that the catalytic unit of 140,000 M.W. should contain two active sites. However phosphorylation of the active site histidine residue leads to incorporation of only 1.0 - 1.5 moles of  $P_i$  per mole of enzyme. This discrepancy can be explained by a negative co-operativity between the proposed active sites (Chapter V). However it remains possible that there are small differences in subunits, not detectable on polyacrylamide gel electrophoresis, such that there would in fact be only one active site per enzyme molecule. Although this possibility is unlikely, a



determination of the number of substrate binding sites per 140,000 M.W. unit would be of interest in relation to the proposed subunit structure.

Binding studies with this enzyme have proven technically difficult due to the lability of the dephospho-enzyme. As a result of this problem, equilibrium dialysis studies of substrate binding to this form of the enzyme have not proven to be possible. In addition, the tendency of ATP to phosphorylate the enzyme even in the absence of  $Mg^{++}$  precludes study of its binding to the dephospho-enzyme. Equilibrium dialysis is possible with the phosphorylated enzyme, but in this case the tendency of some substrates to dephosphorylate the enzyme (42, see also Chapter V) limits the range of the studies.

The preliminary results considered in this Chapter represent two approaches to the problem. One concerns conventional equilibrium dialysis studies using  $^3H$ -succinate binding to the phosphorylated enzyme in the presence of EDTA to prevent dephosphorylation. The other approach used is the rate of dialysis technique developed by Colowick and Womack (46). The binding of  $^3H$ -ADP to dephosphorylated enzyme was studied in this case. The effect of  $P_i$  and succinate on the fluorescent spectrum of SCS has also been investigated.

#### B. Results and Discussion

Figure 29 is a Scatchard plot representing equilibrium dialysis studies of the binding of  $^3H$ -succinate to phosphorylated SCS in the presence of EDTA. The plot indicates that binding does occur with a dissociation constant of 151  $\mu M$ . This value is very similar to the  $K_m$  for succinate, 100  $\mu M$ , determined from initial rate kinetic studies (Table I). This result suggests that  $Mg^{++}$  is not necessary for succinate



binding to E-P and its requirement for the succinate mediated dephosphorylation of E-P (Chapter V) must reflect a more direct involvement in the catalytic reaction.

The value of 0.37 binding sites per enzyme molecule is difficult to interpret because of the variability of specific activity of SCS preparations and the uncertainty concerning the stoichiometry of phosphorylation of active sites. The enzyme used in this study had a specific activity of 34.3 units/mgm. Samples phosphorylated under the conditions given in Table II incorporated 0.8 moles of phosphate per mole of enzyme from ATP- $\gamma$ -<sup>32</sup>P. If succinate binds to only phosphorylated sites it might be expected to show a similar n value, assuming that all phosphorylated sites are identical. This result will be discussed again in conjunction with that for <sup>3</sup>H-ADP binding, following.

Figure 30 is a Scatchard plot for <sup>3</sup>H-ADP binding to the dephospho-enzyme measured by the rate of dialysis technique. The dissociation constant for ADP is 68  $\mu$ M which is somewhat higher than the  $K_m$  of 12  $\mu$ M from initial rate kinetic studies, although they are of the same order of magnitude.

The number of active sites per enzyme molecule for ADP binding is calculated as 1.43. This is again difficult to interpret due to the instability of the enzyme and uncertainties as to maximum specific activities. The specific activity of the enzyme used here is 32.4 units/mgm while values as high as 45 have been observed for this enzyme. Thus if ADP binds only to active enzyme, a value of 2





Figure 29. Binding of  $^3\text{H}$ -succinate to phosphorylated SCS by equilibrium dialysis. Dialysis cells contained 88  $\mu\text{M}$  SCS (S.A. = 34.3), 10 mM EDTA, and 50 mM Tris-HCl, 50 mM KCl, pH 7.2, in a volume of 90  $\mu\text{l}$  in one chamber.  $^3\text{H}$ -succinate was present in the other chamber at concentrations from 0.025 mM to 0.4 mM. Data are expressed as  $r$ ,  $\mu\text{moles}$  of substrate bound per  $\mu\text{mole}$  of SCS and are plotted as  $r/[\text{free succinate}]$  versus  $r$ .

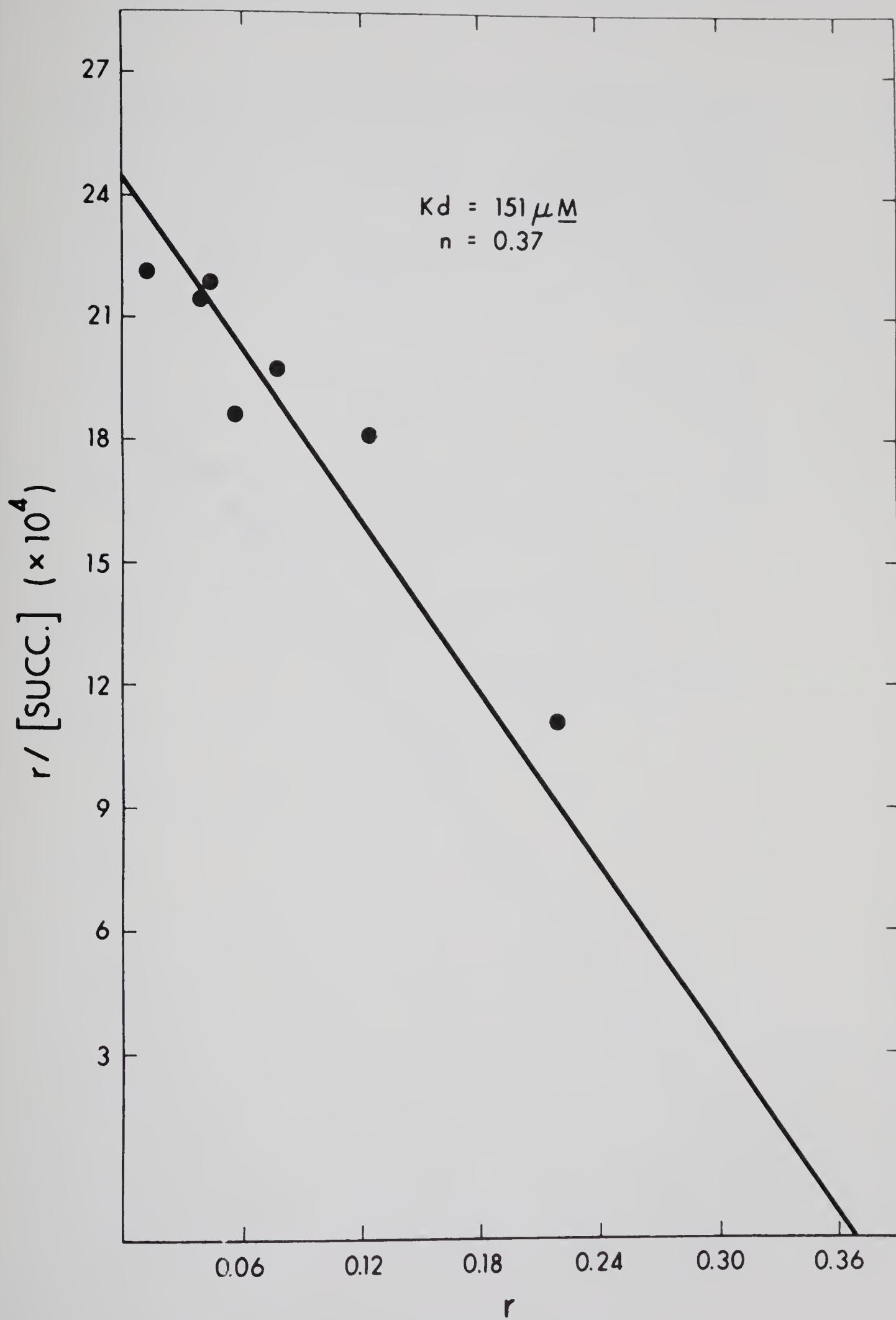
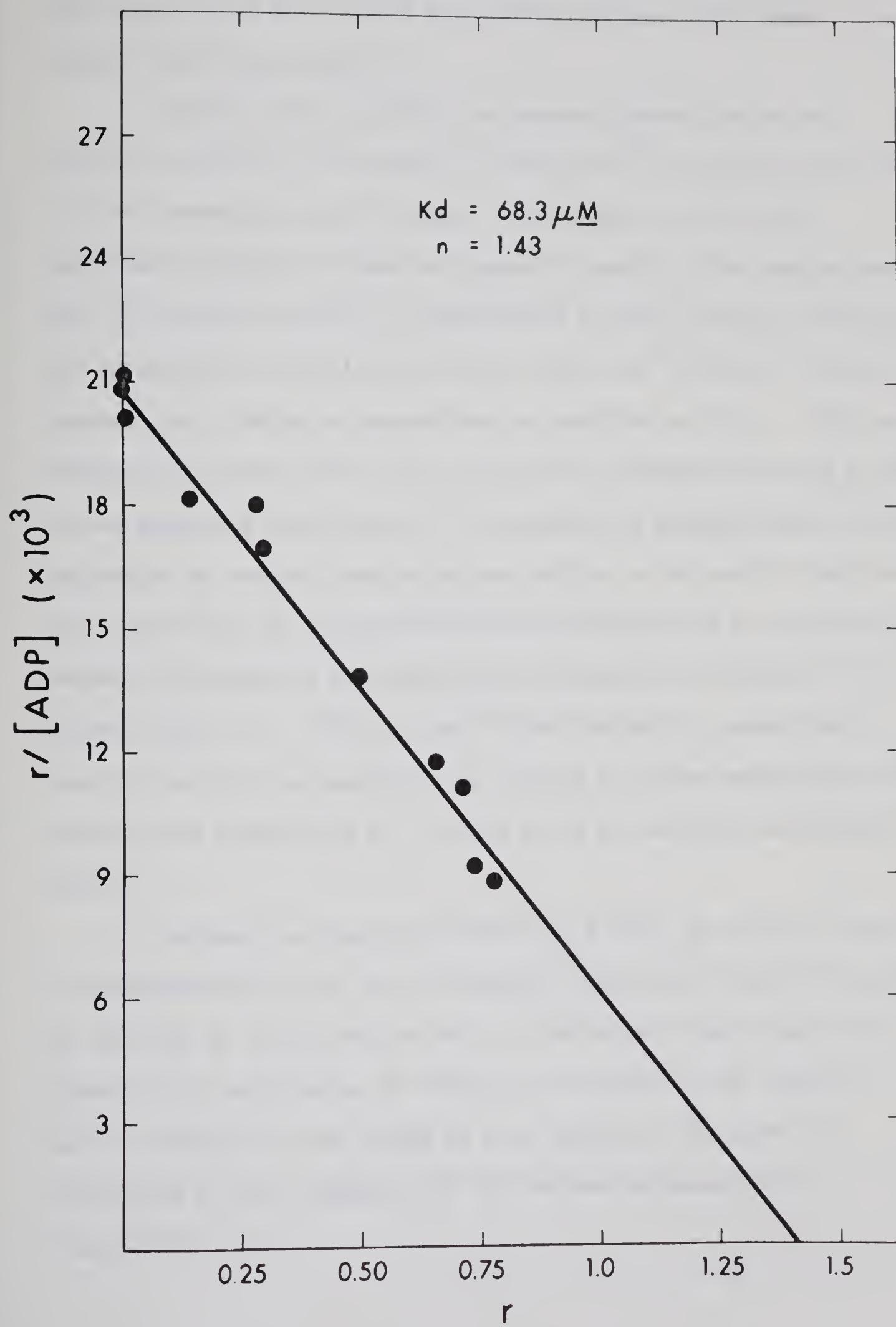






Figure 30. Binding of  $^3\text{H}$ -ADP to SCS by rate of dialysis measurements.

Dialysis flow cell contained 70  $\mu\text{M}$  SCS (S.A. = 32.4), 10 mM  $\text{MgCl}_2$ , 50 mM Tris-HCl, 50 mM KCl, pH 7.2 in a volume of 1 ml in the upper chamber. Flow rate of buffer (50 mM Tris-HCl, 50 mM KCl, pH 7.2) through the lower chamber was 3.3 ml/min. Experiments were initiated by adding 0.1  $\mu\text{M}$   $^3\text{H}$ -ADP to the upper chamber, followed by unlabelled ADP in increments from 20  $\mu\text{M}$  to 200  $\mu\text{M}$  as described under experimental procedures (Chapter II). Data are expressed as  $r$ ,  $\mu\text{moles of substrate bound per }\mu\text{mole of SCS}$  and are plotted as  $r/[\text{free ADP}]$  versus  $r$ .





ADP-binding sites per 140,000 M.W. enzyme molecule gains some support from these results.

Table V shows the effect of protein concentration and specific activity on the amount of binding of  $^3\text{H}$ -succinate and  $^3\text{H}$ -ADP at fixed concentrations of ligand. The points are taken from experiments similar to those of figures 29 and 30. The results show that  $^3\text{H}$ -succinate binding is proportional to total protein concentration and independent of specific activity, while for  $^3\text{H}$ -ADP the results suggest that binding is proportional to specific activity. This would support the concept that in the system with phosphorylated SCS + EDTA we are measuring the binding of  $^3\text{H}$ -succinate at phosphorylated sites regardless of whether these sites are active in the overall reaction. This possibility is consistent with the observed lack of correlation between the degree of phosphorylation and specific activity of this enzyme (Table II). The fact that  $^3\text{H}$ -ADP binding is proportional to specific activity is suggestive of binding to active enzyme only and supports the possibility of 2 active sites per molecule as mentioned above.

Attempts to study the binding of  $[^{32}\text{P}]P_i$  as well as  $^3\text{H}$ -succinate to dephospho-SCS by the rate of dialysis method have been unsuccessful. In the case of  $P_i$  this may be due to a relatively high dissociation constant (1.2 mM, Chapter V) while for succinate it may reflect a lack of binding of this ligand to free enzyme in the absence of saturating  $P_i$  as is suggested by the isotope exchange results (Chapter IV).



TABLE V

Effect of Specific Activity on Binding of  
Succinate and ADP to Succinyl CoA Synthetase

$^3\text{H}$ -succinate Binding

Conc. [E] ( $\mu\text{M}$ )	Specific Activity (units/mgm)	B/[E]
60	40	0.037
86	22	0.039
96	34	0.043

$^3\text{H}$ -ADP Binding

Conc. [E] ( $\mu\text{M}$ )	Specific Activity (units/mgm)	B/[E]
68.2	32.4	0.47
68.2	30.2	0.45
111.0	20.4	0.25

Points at a constant concentration of substrate are taken from 3 experiments of the type reported in figures 29 and 30.

$^3\text{H}$ -succinate concentration = 0.04 mM.

$^3\text{H}$ -ADP concentration = 0.06 mM.

Succinate binding measured by equilibrium dialysis.

ADP binding measured by rate of dialysis.

B/[E] = concentration bound substrate/total enzyme concentration.



Dissociation constants so far determined are compared with  $K_m$  values in Table VI. The constants are generally in the same range and thus do not offer an explanation of the preferred pathway in the formally random kinetic mechanism. However, these results are clearly preliminary. They do serve to point out the problems inherent in binding studies in this system. A clear definition of the relationship of specific activity and phosphorylation as well as development of conditions in which the dephospho-enzyme is more stable would make possible much more definitive conclusions from this type of experiment.

Figure 31 is a fluorescent spectrum of SCS excited at 280 nm. The fluorescent emission peak is due to enzyme tryptophan residues and occurs at 334 nm. Tryptophan fluoresces at about 350 nm in water and the wavelength for SCS emission suggests that most tryptophan residues in this enzyme are located in rather hydrophobic environments.

The successive decreases in emission peak height in figure 31 are in the presence of 10 mM and 20 mM  $P_i$  respectively. The changes are quite small, a 2% decrease in peak height at maximum and might be explained by a possible quenching effect of phosphate ion. Such a change is nevertheless consistent with the possibility of a conformational change on  $P_i$  binding as suggested by trypsin-catalyzed inactivation studies. Other spectra appear to show a similar difference between dephospho-enzyme and phosphorylated enzyme emission peaks although the comparison of such small differences between different enzyme samples is difficult. The addition of succinate at a concentration of 2 mM to dephospho-SCS resulted in no significant change in emission spectrum.



TABLE VI

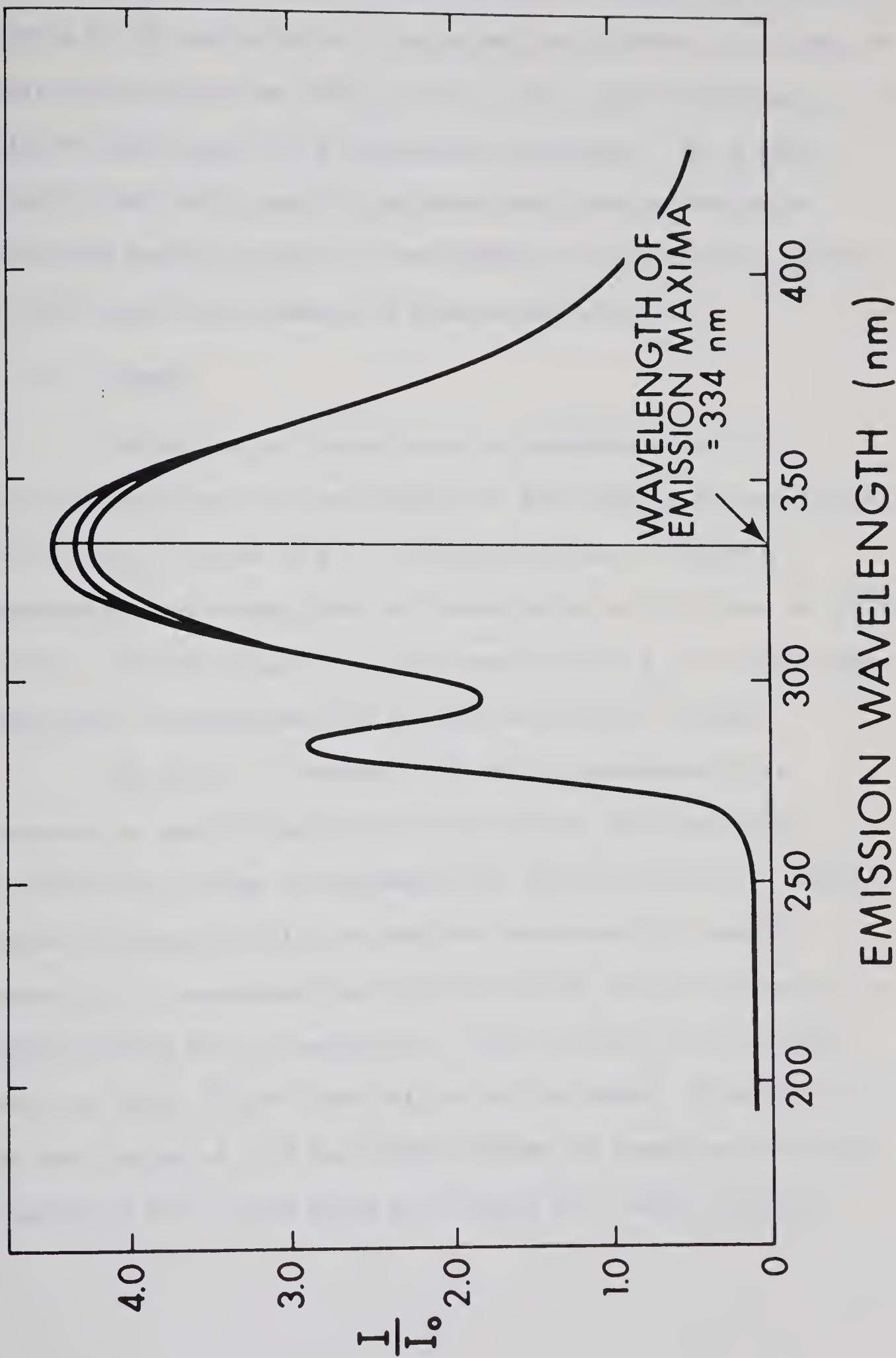
Dissociation and Michaelis Constants for Substrates of SCS

Substrate	$K_m$ (M)	$K_d$ (M)
ATP	$2 \times 10^{-5}$	
Succ	$1 \times 10^{-4}$	$1.5 \times 10^{-4}$
CoA	$1.5 \times 10^{-6}$	
ADP	$1.2 \times 10^{-5}$	$6.8 \times 10^{-5}$
P <sub>i</sub>	$2.6 \times 10^{-3}$	$1.2 \times 10^{-3}$
Succ-CoA	$7.7 \times 10^{-6}$	





Figure 31. Fluorescent spectrum of SCS and effect of  $P_i$  on the spectrum. Cuvette contained 0.154 mgm/ml of dephospho-SCS, 10 mM  $MgCl_2$ , and 50 mM Tris-HCl, pH 7.2 in a volume of 2 ml at 25°C. Maximum peak height at 334 nm is the spectrum of dephospho-SCS. Successive reductions in peak height occur upon addition of 10 mM and 20 mM  $P_i$  respectively.  $I/I_o$  represents relativity intensity of emission with respect to an arbitrary zero. Peak at 280 nm is due to the initial excitation at this wavelength.





The small effect or lack of effect of substrates on these spectra is not unexpected as this enzyme has a rather low content of tryptophan and tyrosine ( $OD_{280} = 0.511$  (12)) and the wavelength of the emission peak suggests the tryptophans are buried. It is quite possible that small specific conformational changes induced by substrates would not alter the environment of such residues enough to cause significant changes in fluorescent spectra.

### C. Summary

The binding of  $^3H$ -succinate to phosphorylated SCS by equilibrium dialysis in the presence of EDTA gives a K dissociation of 151  $\mu M$  and an n value of 0.37. Binding studies of  $^3H$ -ADP to dephosphorylated enzyme gives a K dissociation of 68  $\mu M$  and an n value of 1.43. The Kd values are in the same range as  $K_m$  values for these substrates as determined from initial rate kinetic studies.

The amount of binding of  $^3H$ -ADP to dephospho-SCS is dependent on specific activity, while that of succinate to the phosphorylated enzyme is independent of specific activity. Since the degree of phosphorylation is also not correlated with specific activity it is suggested that succinate binds in this system only to phosphorylated sites, regardless of their activity in the overall reaction, while  $^3H$ -ADP binds only to active enzyme. This observation and the n value of 1.43 for  $^3H$ -ADP binding are consistant with the possibility of 2 active sites per 140,000 M.W. enzyme molecule.



Excitation of SCS at 280 nm gives a fluorescent emission peak at 334 nm.  $P_i$  has a slight effect on peak height in harmony with previous observations that indicate it may cause a conformational change on binding. Phosphorylation of the enzyme also appeared to alter the emission spectrum slightly while addition of succinate had no detectable effect.



## CHAPTER VII

## CONCLUSION

The initial rate kinetic studies of SCS are not consistent with a ping-pong kinetic mechanism despite the participation of a covalent enzyme-phosphate intermediate in the reaction. These studies, and studies of the kinetics of ATP  $\longleftrightarrow$  ADP exchange, can be interpreted in support of a random sequential mechanism with a preferred pathway involving initial ATP addition and final ADP dissociation from the enzyme. This result is compatible with the observations of substrate synergism with this enzyme which imply that the overall reaction does not proceed near its maximal rate until all substrate-binding sites are occupied.

The study also demonstrates a possible danger in the use of initial rate kinetic data as the sole test of mechanism. Since enzymes involving covalent intermediates have generally shown ping-pong kinetics, a tendency may arise to consider the lack of such kinetics as evidence against these intermediates. A consideration of the theory of initial rate kinetics shows that this type of evidence is not valid, and these studies on SCS represent a clear case of an enzyme which is known to involve a covalent enzyme-substrate intermediate in catalysis that shows sequential rather than ping-pong kinetics.

Also because of their inherent limitations, kinetic studies such as these cannot distinguish between the concerted mechanism proposed by Robinson et al. (25) and the participation of an enzyme-bound succinyl-phosphate intermediate as has been advocated by



Nishimura (30,35) and by Spector (31). The evidence for an enzyme-bound succinyl-phosphate is, however, extensive, lacking only kinetic confirmation, and it appears likely that this species is an intermediate in the reaction.

The ability of substrates to stimulate partial reactions in which they have no direct role can be interpreted in terms of the induced-fit model of Koshland (65). Thus, for SCS, the presence of all substrates in a quaternary complex would induce an optimum conformation for reaction. In this study, no effect of substrates, other than  $P_i$ , on the rate of trypsin-catalyzed inactivation or on the fluorescent spectrum of SCS was noted. As pointed out previously (Chapter V), this does not mean that no conformational changes occur upon binding of substrates, as small changes may not be detectable by these means. It is also possible that other microscopic effects of substrates, for example on the effective net charge at the active site, might contribute to the synergistic effect.

The trypsin inactivation studies and the fluorescent spectra are consistent with a conformational change accompanying both the phosphorylation of the active site histidine and the binding of inorganic phosphate to the enzyme. This supports the possibility of a negative co-operativity between active sites as an explanation of the difference between the degree of phosphorylation normally obtainable with this enzyme and the apparent subunit structure as detailed previously (Chapter I, Chapter V). Other enzymes have been found to display this type of relationship between active sites, which Koshland has called "half of the sites reactivity" (44). He gives a list of



eleven enzymes for which there is evidence of this phenomenon. The list includes alkaline phosphatase of E. coli, which displays an apparent negative co-operativity between active sites in conjunction with the formation of an active site phosphoserine (71,72). This phospho-enzyme species is also known to be an intermediate in the alkaline phosphatase reaction (74,75).

It is possible to speculate that a conformational change upon the formation of a covalent enzyme-substrate intermediate may have a mechanistic function at the active site. As discussed by Koshland (73), with an enzymic reaction involving a covalent enzyme substrate intermediate, the enzyme must be a better attacking group than the final acceptor and a better leaving group than the leaving group on the initial donor. In most cases enzyme substrate intermediates have been found to be chemically similar to the donor compounds and thus the chemical natures of these intermediates do not appear to be the source of the catalytic power of the enzyme. However, a conformational change at the active site could explain the ability of the enzyme to be both a good attacking and leaving group in a similar chemical reaction. That is, the free enzyme active site may be constructed in such a way that attack on the substrate and formation of the E-S covalent intermediate is favoured. The intermediate might then undergo a conformational change that would align the active site to favour the breakage of the E-S bond.

The conformational change observed upon phosphorylation of SCS in these studies is in keeping with this explanation. It is also interesting to note that Wilson et al. (76) have proposed a scheme for



alkaline phosphatase action at pH 8.0 that involves an equilibrium between an  $E_\alpha$  and  $E_\beta$  form of the enzyme. The  $E_\alpha$  form cannot be phosphorylated by substrate, while phosphorylation of  $E_\beta$  is proposed to lead to a conformational change giving  $E_\alpha\text{-P}$ . This scheme is consistent with that proposed above with  $E_\alpha$  being the "leaving group" conformation of the enzyme and  $E_\beta$  being the "attacking group" form. If a conformational change of the type proposed was propagated symmetrically to identical subunits it would lead to a "half of the sites reactivity" phenomenon as is observed with both of these enzymes. While this proposal does provide an explanation of some of the observations made concerning the phosphorylation of SCS, it is at present highly speculative. A study of transient kinetics and the use of stop-flow relaxation techniques to observe possible rearrangements of free enzyme and enzyme-substrate complexes and locate the rate limiting step of the reaction would be of interest in the evaluation of the proposal.

The instability of free SCS and the apparent ability of enzyme inactive in the overall assay to incorporate phosphate makes the accurate estimation of the amount of active enzyme in a preparation very difficult. Thus the interpretation of the binding studies obtained here is also difficult. Nevertheless, the results for ADP binding are consistent with two active sites per 140,000 M.W. tetramer. As mentioned previously, the development of conditions wherein the dephosphorylated enzyme is stable is imperative to allow a more complete study of substrate binding. The separation of subunits in quantities to allow for an investigation of their properties would also be of interest. The proposed  $\alpha_2\beta_2$  structure, in conjunction with the phosphorylation data



(43), suggests the possibility of catalytic and regulatory subunits, although no regulatory mechanisms are known for this enzyme. Another possible role for this type of quaternary structure might involve a total active site constructed by contributions from both subunits, with at least some substrate-binding sites occurring in the large subunit, and phosphoprotein formation occurring in the smaller subunit species. Further investigation of this and other phosphohistidine-containing enzymes are required to help clarify this point.



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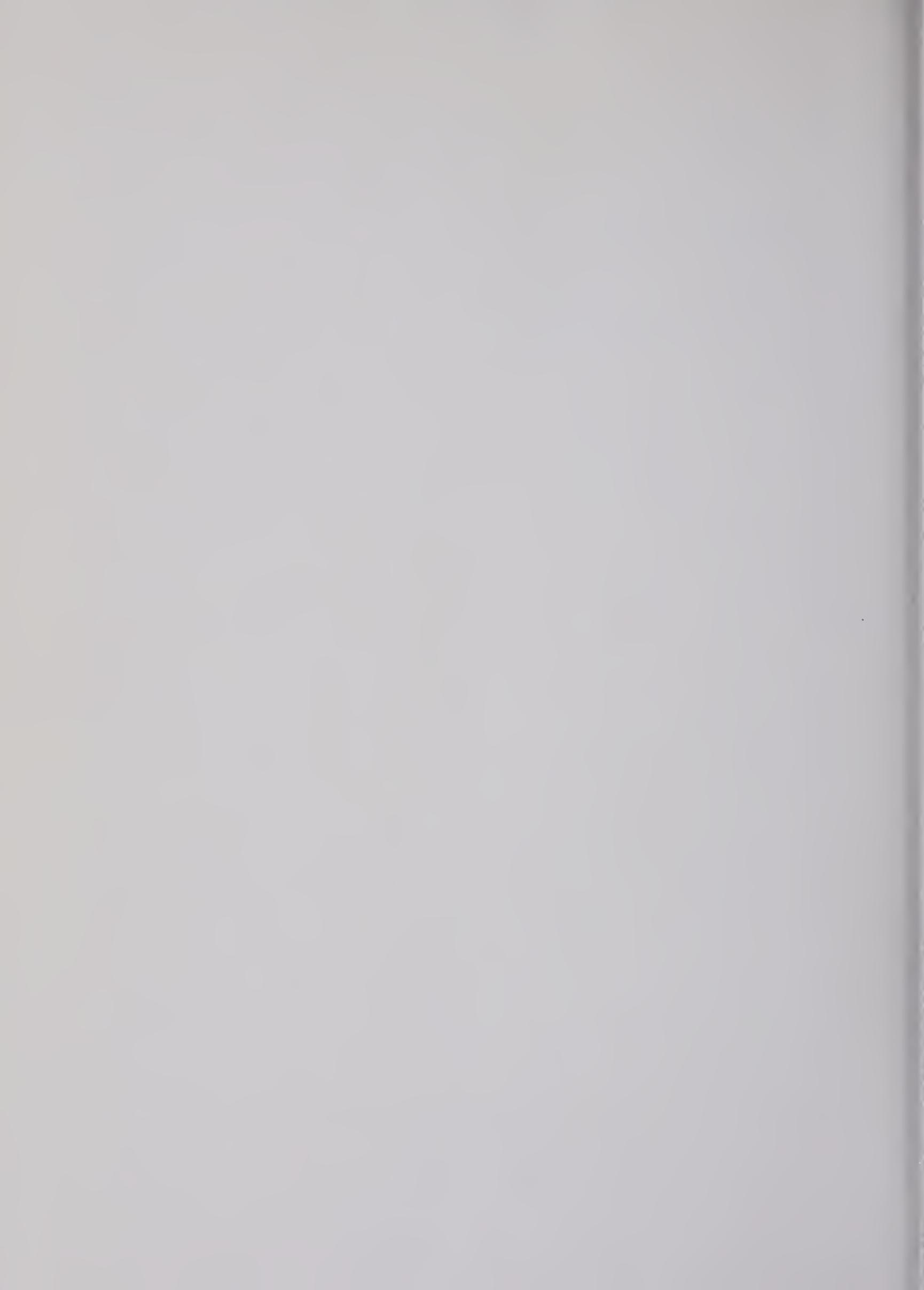
## APPENDIX

Publication arising from this work:

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